(54) Title: ANTIBODIES THAT RECOGNIZE APP CLEAVED BY CASPASES AND METHODS OF USE
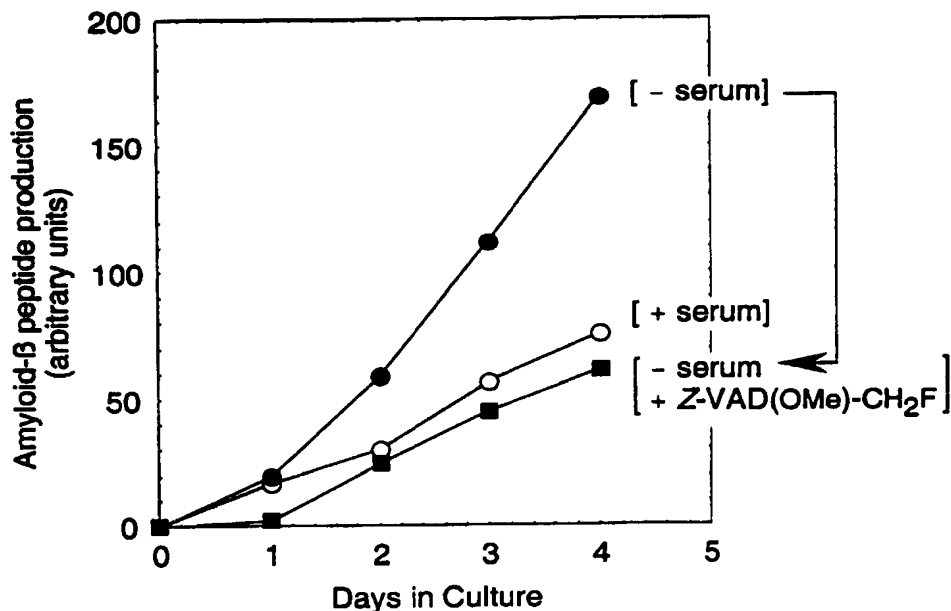
(57) Abstract

Amyloid–β peptide (Aβ) formation and the apoptotic loss of neurons are intimately linked processes in the pathogenesis of neurodegenerative diseases, particularly Alzheimer's disease. The roles of apoptotic proteases and caspases in cleavage of amyloid–β precursor protein (APP) and biogenesis of amyloidogenic Aβ peptide species are described. Antibodies that recognize caspase cleaved APP are described as well as various utilities therefore.

TITLE OF THE INVENTION
ANTIBODIES THAT RECOGNIZE APP CLEAVED BY CASPASES AND
METHODS OF USE

5   BACKGROUND OF THE INVENTION

The present invention is directed towards novel antibodies capable of recognizing neo-epitopes exposed following caspase mediated cleavage of amyloid-β precursor protein and uses thereof.

The loss of hippocampal neurons by apoptotic cell death is a prominent feature of Alzheimer's disease (Cotman and Su, 1996; Li et al., 1997; Smale et al., 1995; Su et al., 1997). One potential factor contributing to the susceptibility of these cells to premature death arises from the cytotoxic effects of amyloid-ß peptide deposition at or near sites of neuronal degeneration. Cultured human cells, including neurons, undergo apoptotic cell suicide when treated with amyloidogenic Aß peptides (Forloni et al., 1996; LaFerla et al., 1995; Loo et al., 1993). At the same time, neurons undergoing apoptosis generate elevated levels of cytotoxic Aß peptide species (LeBlanc, 1995). A possible scenario in the pathogenesis of Alzheimer's disease, therefore, is that the escalated Aß peptide formation that occurs as a consequence of genetic predisposition or other physiological factors provokes partial activation of the endogenous cell suicide pathway in susceptible neurons. The sensitized cells, in turn, produce elevated levels of the Aß peptide, leading to exacerbation of a vicious cycle, which culminates in the progressive neuronal loss that is the hallmark of Alzheimer's disease. For this to be true, components of the apoptotic machinery must contribute either directly or indirectly to the complex proteolytic processing (Haass and Selkoe, 1993; Selkoe et al., 1996; Sisodia and Price, 1995) of the amyloid-ß precursor protein (APP). Proteolytic processing of APP leads to elevated Aß peptide formation and to other APP protease cleavage products observed in apoptotic cells. In this study the role of apoptotic proteases, specifically the caspases, in APP processing and in the biogenesis of amyloidogenic Aß peptide species is provided.

To date there are no direct or indirect methods for specifically evaluating the presence of APP caspase cleavage products. Therefore it would be useful to develop agents that can specifically detect the presence of APP caspase cleavage products. The presence of the cleavage products can be used as an indication of neuronal apoptosis. In particular, such agents would be useful in recognizing neuronal degeneration, the presence neurodegenrative diseases and in

evaluating the progression of such diseases in mammals. More particularly, such agents can be used as a tool in evaluating the effect of candidate inhibitors on neuronal apoptosis and as a possible tool in diagnosing neurodegenerative diseases.

5　　　　　Consequently, one object of the present invention was to develop agents capable of specifically detecting APP protease cleavage products.

Another object was to identify neuronal apoptosis using the agents of the invention.

An additional object was to use the agents of this invention to distinguish neuronal apoptosis from caspase-independent neuronal necrotic cell death.

10　　　　　A further object was to use the agents of this invention to detect neuronal apoptosis as an indicator of a neurodegenerative condition and brain injury.

These and other objects will become apparent to those of ordinary skill in the art from the teachings provided herein.

The application refers to a number of publications, the content of

15　　which is hereby incorporated by reference in their entirety.

SUMMARY OF THE INVENTION

The present invention is directed to an antibody that recognizes a neo-epitope created or exposed following a caspase mediated cleavage of APP or APLP

20　　and to uses thereof.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention is further described in connection with the drawings appended hereto, in which:

25　　　　　**Figure 1A** shows that caspase inhibition ablates escalated amyloid-ß peptide production in apoptotic neuronal cells. Retinoic acid-differentiated neuronal NT2 cells (hNT) were cultured in the presence (○) or absence (● ,■) of serum for 4 days. Half of the serum-deprived cell cultures were treated with Z-VAD(OMe)-CH_2F (100 μM initial dose, 50 μM addition at 24 hr intervals) (■). Aß (1-40) was measured

30　　at the indicated intervals in the resulting cell culture supernatants by sandwich ELISA. The relative rates of Aß production were calculated once a steady state was achieved (in this case, the slope of the values from days 2-4). A representative experiment (n = 2) is shown and each data point is the average value from triplicate cultures.

Figure 1B shows the caspase-associated APP proteolysis during

35　　apoptosis. NT2 cells were induced to undergo apoptosis with 1 μg/ml camptothecin

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for 6 hr in the absence or presence of 33 μM Z-VAD(OMe)-CH$_2$F as indicated. Apoptosis was assessed by oligonucleosomal DNA fragmentation (left panel). Cell lysates were prepared using urea/SDS, resolved on polyacrylamide gels, then immunoblotted for caspase-3 (right, upper panel), poly(ADP)-ribose polymerase (right, middle panel) or APP (right, lower panel). A representative experiment ($n = 5$) is shown.

Figure 1C shows the proteolytic cleavage of amyloid-ß precursor protein by group II caspases in apoptotic cell extracts. [^{35}S]Met-labeled APP was generated by coupled *in vitro* transcription/ translation then incubated with cytosolic extracts from healthy Jurkat cells (lane 1) or extracts from cells that were induced to undergo apoptosis by CD-95 (Fas, APO-1) immuno-ligation (lane 2). Intact APP (p120^{APP}) was cleaved to three discrete intermediates (APP) of approximately 85, 88 and 90 kDa. A representative example ($n = 4$) is shown.

Figure 1D shows the proteolytic cleavage of amyloid-ß precursor protein by group II caspases in apoptotic cell extracts. The cleavage of [^{35}S]Met-labeled APP in apoptotic Jurkat extracts was tested in the presence of the indicated concentrations of tetrapeptide-aldehyde caspase inhibitors (Ac-YVAD-CHO, ■; Ac-DEVD-CHO, O; Ac-IETD-CHO, ●) and quantified by laser densitometry of the resulting fluorogram. A representative example ($n = 2$) is shown.

Figure 1E shows the cleavage of amyloid-ß precursor protein by recombinant caspase-3. [^{35}S]Met-labeled APP was incubated with the indicated concentrations of recombinant human caspase-3 for 60 min at 37°C. APP cleavage was quantified by laser densitometry and used to determine values for k_{cat}/K_m from the relationship $S_t/S_o = e^{-kobs*t}$ where S_t is the concentration of substrate (APP) remaining at time t, S_o is the initial substrate concentration, and $k_{obs}=k_{cat}*$[caspase-3]/K_m. A representative experiment ($n = 2$) is shown.

Figure 2 shows increased caspase-3 immunoreactivity in dying pyramidal neurons of CA3 region of Alzheimer's brain hippocampus. Paraffin-embedded sections were processed for antigen retrieval and peroxidase neutralization then probed with an immunoaffinity-purified antibody raised against the large subunit of human caspase-3 (MF-R280) followed by anti-rabbit-HRP visualization (Vectalabs, UK) and hematoxylin counterstaining. The staining was specific as judged by: a) inability of the antiserum to cross react with any other human caspase; b) absence of staining with pre-immune serum; c) ability of antigen pre-adsorption to

abolish immunostaining; and d) similarity with results using antibodies derived from another rabbit (not shown). The width of each panel corresponds to 140 μm. Identical results were found in brains from other donors (3 Alzheimer's, 2 controls). The examples shown are from a 82 yr old female with clinical and post-mortem

5   diagnosis of Alzheimer's disease and an 88 yr old male with no clinical abnormalities who died of a pulmonary embolus and whose brain was judged to be neurologically normal.

Figure 2A shows the paraffin-embedded sections from the age-matched neurologically normal individual. The white arrows indicate healthy,

10   caspase-3-negative pyramidal neurons showing little, if any, caspase-3 immunoreactivity.

Figure 2B shows the paraffin-embedded sections from the Alzheimer's patient. Black arrows indicate caspase-3 positive immunoreactive neurons (brown) in an area susceptible to degeneration and having degenerative

15   morphology (including 'ghosts') in the Alzheimer's sample.

Figure 3A shows the identification and mutagenic elimination of sites of caspase-mediated proteolysis of amyloid-ß precursor protein. The three potential caspase cleavage sites were identified by $[^{35}S]$Cys versus $[^{35}S]$Met differential mapping and mass analysis of caspase-cleaved ΔN-APP deletion contructs (not

20   shown). The putative sites were then confirmed by sequential elimination of the predicted P_1 Asp residues by site-directed mutagenesis (a P_1 Asp is essential for caspase recognition). The indicated $[^{35}S]$Met APP mutant polypeptides were generated by coupled *in vitro* transcription/ translation then incubated in the absence (lanes 1,3,5,7) or presence (lanes 2,4,6,8) of 8 nM caspase-3. A representative

25   example ($n = 3$) is shown.

Figure 3B shows a pictogram of the location of caspase cleavage sites within the amyloid-ß precursor protein in relation to other structural features. Domains and interaction sites within the amyloid-ß precursor protein are summarized. The three sites of caspase proteolysis are indicated on the bottom with the number

30   referring to the Asp residue corresponding to P_1. The region encompassing the Aß peptide is expanded at the top with known mutations indicated in brackets, including the $VKMD^{653}$ - $VNLD^{653}$ 'Swedish' mutation (circled).

Figure 4 shows the caspase-dependent cleavage of the amyloid-ß precursor protein in intact cells during apoptosis and in situ detection of a caspase-

35   generated C-terminal neo-epitope.

Figure 4A shows the C-terminal cleavage of APP during apoptosis in transfected B103 cells. Stable B103 cell lines were generated expressing equivalent amounts of the full-length wild-type APP (wt, lanes 1 & 2), APP lacking Ala^{721}-Asn^{751} - the C-terminal residues following the Asp^{720} caspase cleavage site (ΔC-APP,

5   lanes 3 & 4) - , or full length APP containing a point mutation to eliminate the Asp^{720} caspase cleavage site ($D^{720}A$, lanes 5 & 6). The cell lines were treated for 2 hr in the absence (lanes 1,3,5) or presence (lanes 2,4,6) of 1 μM staurosporine to induce apoptosis. Cells and apoptotic corpses were harvested by centrifugation, washed in PBS and lysed in buffer containing Nonidet P-40. Intact APP was immunodepleted

10   using α-C21 antibody (raised against the C-terminal 21 amino acids of APP) and protein A-Sepharose harvesting. The remaining APP fragments were immunoprecipitated using 6E10 antibody (raised against the first 17 residues of the Aβ peptide) with protein G-Sepharose harvesting. After resolution by SDS-PAGE, the C-terminal-truncated APP fragments were visualized by immunoblotting using

15   22C11 antibody (raised against the amino-terminus of APP). Arrows indicate the migration of the APP product generated by cleavage after Asp^{720} (ΔC-APP) or the equivalent transfection standard (lanes 3 & 4). A representative experiment ($n = 4$) is shown.

Figure 4B shows the specific recognition of a caspase-generated APP

20   neo-epitope by $\alpha\Delta C^{Csp}$-APP. [^{35}S]Met-labeled APP variants were generated by coupled *in vitro* transcription/ translation (upper panel) then immunoprecipitated (lower panel) using a specific antibody ($\alpha\Delta C^{Csp}$-APP) that recognizes the caspase-generated neo-epitope that is exposed following cleavage of APP at Asp^{720}. A representative experiment ($n = 2$) is shown.

25   **Figure 4C** shows the C-terminal cleavage of APP during apoptosis in transfected B103 cells. The B103 stable cell line harboring full-length wild-type APP (described in **Figure 4A**) was treated for 2 hr in the absence (lane 1) or presence (lanes 2 & 3) of 1 μM staurosporine to induce apoptosis. Cells in lane 3 were also treated with 100 μM of the caspase inhibitor, Z-VAD(OMe)-CH$_2$F. Cells and

30   apoptotic corpses were harvested and lysed as described above. Lysates were then immunoprecipitated with $\alpha\Delta C^{Csp}$-APP, resolved by SDS-PAGE and immunoblotted for ΔC-APP using 22C11. As a positive control, untreated B103 cells harboring ΔC-APP by stable transfection were processed the same way (lane 4). A representative experiment ($n = 3$) is shown.

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Figure 4D-4I shows the *in situ* detection of the caspase-generated ΔC-APP neo-epitope during NT2 cell apoptosis. Sub-confluent NT2 cells were induced to undergo apoptosis by incubation for 4 hr with 1 μg/ml camptothecin (E-I) in the absence1 (E, H) or presence (F, I) of 50 μM of the caspase inhibitor, Z-VAD(OMe)-CH$_2$F. Control cells (D, G) were treated the same but with vehicle (DMSO) only. After cell harvesting and fixation, all cells were stained for TUNEL (green) and co-stained using either an antiserum that recognizes the active form of caspase-3 (D, E, F; αCsp-3(MF397); red) or the caspase-generated ΔC-APP neo-epitope (G,H,I; αΔ C^{Csp}-APP; red). All panels are merged images of TUNEL (green) and immunoreactivity (red), yielding yellow where co-incident. A representative experiment ($n = 2$) is shown.

Figure 5 shows the *in vivo* truncation of APP at the C-terminal caspase site during neuronal apoptosis following acute excitotoxic or ischemic injury. The generation of ΔC-APP in hippocampal neurons undergoing apoptosis was examined *in vivo* following systemic administration of the excitotoxin kainic acid (A-F) or transient global cerebral ischemia (G-L).

Figure 5A to 5F shows excitotoxic injury in SV129 mice. The left panels A, C, E are control and the right panels, B, D, F are kainate-treated SV129 mice euthanized, and adjacent brain sections stained for the caspase-generated APP neo-epitope (A and B; αΔC^{Csp}-APP), active caspase-3 (C and D; αCsp-3(MF397)) or TUNEL (E and F). The CA3 region of the hippocampus is shown in all panels at the same magnification (bar in panel F = 100 μ). A representative experiment ($n = 4$) is shown.

Figure 5G to 5L shows the effect of ischemic injury. Transient global ischemia was produced in rats by 12 min of four-vessel occlusion followed by reperfusion for the indicated length of time (control animals (G) underwent sham surgical procudures without ischemia). Sections through the CA1 region of the hippocampus were stained for the caspase-generated APP neo-epitope (G-J, L) or for TUNEL (K). Magnifications for panels G-K are the same (bar in panel K = 400 μ) and panel L is an enlargement (bar = 75 μ) of the region outlined in panel I. A representative example ($n = 4$) is shown.

Figure 6 shows the co-localization of ΔC-APP with amyloid-ß in senile plaques. The bar in panel 6C = 100 μ.

Figure 6A ΔC-APP, visualized by αΔC^{Csp}-APP immunoreactivity, in the same area of the hippocampus as shown in (B).

Figure 6B shows senile plaques identified by amyloid-β immunoreactivity (arrows) in the hippocampus of a patient diagnosed with Alzheimer's disease.

Figure 6C shows the merged image of the caspase-generated APP neo-epitope (red) and Aß (green), yielding yellow where co-incident, illustrates the high degree of overlap between ΔC-APP and amyloid-β immunoreactivities.

Similar results were observed in $n = 7$ clinically-diagnosed Alzheimer's patients (male and female aged 77-91 yrs with 5-14 yrs AD diagnosis; 2.3-4.3 hr post-mortem harvest) but not in 7 age-matched control patients (male and female aged 79-91 yrs, 2.0-4.0 hr post-mortem).

Figure 7 shows that caspase-mediated generation of APP results in elevated amyloid-ß peptide formation. The B103 stable cell lines described for Fig. 4A, expressing comparable levels of full-length APP (wt, columns 1 & 3) or APP lacking Ala^{721}-Asn^{751} (the C-terminal residues following the Asp^{720} caspase cleavage site (αΔC-APP, columns 2 & 4)), were cultured in fresh medium for 20 or 48 hr after which Aß peptide levels in the culture medium were quantified by immunoassay with monoclonal antibodies. A representative experiment ($n = 4$) is shown ± SD.

Figure 8 shows that the 'Swedish' familial mutation improves the ß-secretase target region of APP as a caspase-6 substrate.

Figure 8A shows fluorogenic tetrapeptide-aminomethylcoumarins synthesized to correspond either to the VKMD^{653}/A^{654} site at the ß-secretase target region of APP (VKMD-AMC, O ; see Fig. 3B), the 'Swedish' dipeptide mutation (VNLD-AMC, ●) or the optimal tetrapeptide for caspase-6 predicted by a positional-scanning combinatorial substrate library (VEHD-AMC, ▢). Their suitability as caspase-6 substrates was tested in reaction mixtures containing recombinant human caspase-6 (2 nM) plus varying concentrations of the fluorogenic ligands. A representative experiment ($n = 3$) is shown.

Figure 8B shows the effect of APP constructs generated to eliminate the N-terminal caspase cleavage sites (ΔN-APP) and then engineered to contain either the wild type ß-secretase region (VKMD^{653}/A^{654}; columns 1 & 2), the 'Swedish' mutation (VNLD^{653}/A^{654}; columns 3 & 4) or the 'Swedish' mutation with the P$_1$ Asp that is essential for caspase recognition changed to Ala (VNLA^{653}/A^{654}; columns 5 &

6). [^{35}S]Met-labeled proteins were generated from these constructs by coupled *in vitro* transcription/ translation then incubated with cytosolic extracts from apoptotic cells (solid columns; 1, 3 & 5) or recombinant caspase-6 (hashed columns; 2, 4 & 6). The percentage of the input APP that was cleaved in the ß-secretase target region in

5 60 min was determined by fluorography and is expressed ± SD. The results show an increased susceptibility of the APP ß-secretase site to caspase-6 by the 'Swedish' familial mutation and dependence of elevated amyloid-ß peptide formation in cells harboring 'Swedish' APP on the P_1 Asp necessary for caspase recognition. A representative experiment ($n = 5$) is shown.

10   **Figure 8C** shows a measure of cleavage, [^{35}S]Met-labeled ΔN-APP containing either the wild type ß-secretase sequence (O) or the 'Swedish' dipeptide mutation (●) was incubated with the indicated concentrations of recombinant human caspase-6 for 60 min at 37°C. Cleavage was quantified by phosphorimaging of the ß cleavage fragment and used to determine values for k_{cat}/K_m as described in the legend

15 to Fig. 1F.

         Figure 8D shows immunoreactive products in a stable K562 cell lines generated to harbor full-length APP with the wild type VKMD^{653}/A^{654} site, the 'Swedish' mutation (VNLD^{653}/A^{654}) or the 'Swedish' mutation with the P_1 Asp changed to Ala (VNLA^{653}/A^{654}). Identical quantities of cells, expressing equivalent

20 amounts of the APP constructs, were metabolically labeled by culturing in methionine-free medium for 1 hr then for 5 hr in the presence of [^{35}S]Met. The cells and medium were separated by centrifugation then [^{35}S] Aß peptides were immunoprecipitated from each (using 4G8 antibodies), resolved by SDS-PAGE (10-20% polyacrylamide; Tricine gels) and visualized by fluorography. A representative

25 experiment (n = 3) is shown.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

Unless defined otherwise, the scientific and technological terms and
5   nomenclature used herein have the same meaning as commonly understood by a
person of ordinary skill to which this invention pertains. Generally, the procedures
for cell culture, infection, molecular biology methods and the like are common
methods used in the art. Such standard techniques can be found in reference manuals
such as for example Sambrook *et al.* (1989, Molecular Cloning - A Laboratory
10   Manual, Cold Spring Harbor Laboratories) and Ausubel *et al.* (1994, Current
Protocols in Molecular Biology, Wiley; New York).

Generally the procedures for immunology such as, generation of
antibodies, animal immunization, ELISA and the isolation of pure antibodies by
means including affinity chromatography are common methods used in the art. Such
15   standard techniques can be found in reference manuals such as for example Harlow et
al. (1988, Antibodies - A Laboratory Manual, Cold Spring Harbor Laboratories)

Nucleotide sequences are presented herein by single strand, in the 5' to
3' direction, from left to right, using the one letter nucleotide symbols as commonly
used in the art and in accordance with the recommendations of the IUPAC-IUB
20   Biochemical Nomenclature Commission (Biochemistry, 1972, 11:1726-1732).

The present description refers to a number of routinely used
recombinant DNA (rDNA) technology terms. Nevertheless, definitions of selected
examples of such rDNA terms are provided for clarity and consistency.

The term "recombinant DNA" or "recombinant plasmid" as known in
25   the art refers to a DNA molecule resulting from the joining of DNA segments. This is
often referred to as genetic engineering.

The term "DNA segment or molecule or sequence", is used herein, to
refer to molecules comprised of the deoxyribonucleotides adenine (A), guanine (G),
thymine (T) and/or cytosine (C). These segments, molecules or sequences can be
30   found in nature or synthetically derived. When read in accordance with the genetic
code, these sequences can encode a linear stretch or sequence of amino acids which
can be referred to as a polypeptide, protein, protein fragment and the like.

As used herein, the term "gene" is well known in the art and relates to
a nucleic acid sequence defining a single protein or polypeptide. The nucleic acid can

be full-length or a partial sequence encoding a polypeptide, so long as the functional activity of the polypeptide is retained.

A "structural gene" defines a DNA sequence transcribed into RNA and translated into a protein having a specific amino acid sequence thereby giving rise to a

5   specific polypeptide or protein.

"Restriction endonuclease or restriction enzyme" is an enzyme that has the capacity to recognize a specific base sequence (usually 4, 5 or 6 base pair in length) in a DNA molecule, and to cleave the DNA molecule at every place where this sequence appears. An example of such an enzyme is *EcoRI*, which recognizes the

10   base sequence GAATTC/CTTAAG and cleaves a DNA molecule at this recognition site.

"Restriction fragments" are DNA molecules produced by the digestion of DNA with a restriction endonuclease. Any given linear genome or DNA segment can be digested by a particular restriction endonuclease into at least two discrete

15   molecules of restriction fragments.

"Agarose gel electrophoresis" is an analytical method for fractionating double-stranded DNA molecules based on the size of the DNA. The method is based on that DNA molecules migrate through a gel as through a sieve, whereby the smallest DNA molecule has the greatest mobility and travels the farthest through the

20   gel. The sieving characteristics of the gel retards the largest DNA molecules such that, these have the least mobility. The fractionated DNA can be visualized by staining the gel using methods well known in the art, nucleic acid hybridization or by tagging the fractionated DNA molecules with a detectable label. All these methods are well known in the art, specific methods can be found in Ausubel *et al. (supra)*.

25   "Oligonucleotide or oligomer" is a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, preferably more than three. The exact size of the molecule will depend on many factors, which in turn depend on the ultimate function or use of the oligonucleotide . An oligonucleotide can be derived synthetically, by cloning or by amplification.

30   "Sequence amplification" is a method for generating large amounts of a target sequence. In general, one or more amplification primers are annealed to a nucleic acid sequence. Using appropriate enzymes, sequences found adjacent or in between the primers are amplified. An amplification method used herein is the polymerase chain reaction (PCR).

"Amplification primer" refers to an oligonucleotide, capable of annealing to a DNA region adjacent to a target sequence and serving as the initiation primer for DNA synthesis under suitable conditions well known in the art. The synthesized primer extension product is complementary to the target sequence.

5   The terms "plasmid" or "vector" are commonly known in the art and refer to a genetic vehicle, including but not limited to plasmid DNA, phage DNA, viral DNA and the like, which can incorporate the nucleotide sequence, or sequences of the present invention and serve as a DNA vehicle into which DNA of the present invention can be cloned. Numerous types of vectors exist and are well known in the

10   art.

The term "DNA construct" as used herein refers to a vector or plasmid comprising a cloned nucleotide sequence.

The term "expression" defines the process by which a structural gene is transcribed into mRNA (transcription), the mRNA is then translated (translation) into one

15   polypeptide (or protein) or more.

The terminology "expression vector" defines a vector or vehicle as described above that is designed to enable the expression of an inserted sequence following transformation into a host. The cloned gene(s) (inserted sequence), usually placed under the control of control element sequences such as promoter sequences

20   initiates the transcription of the inserted sequence. Such expression control sequences will vary depending on whether the vector is designed to express the operably linked gene in a prokaryotic or eukaryotic host or both (shuttle vectors) and can additionally contain transcriptional elements such as enhancer elements, termination sequences, tissue-specificity elements, and/or translational initiation and termination sites.

25   By "eukaryotic expression system" is meant the combination of an appropriate expression vector and an eukaryotic cell line, which can be used to express a protein of interest. In some systems the gene encoding the protein may be inserted into the genome of a virus which can infect a particular host cell. Plasmid vectors containing the desired gene may also be used. In all cases, the vector will

30   contain appropriate control elements (promoter) to express protein in the host cell. Additional components, for example a vector or viral genome coding for T7 polymerase, may also be necessary in certain expression systems. Eukaryotic cell types typically used are yeast (e.g. *Saccharomyces cerevisiae, Pischia pastoris*) transfected with a plasmid vector; insect cells (e.g. SF9, SF21) infected with

35   baculovirus (*Autographa californica or Bombyx mori*) (Luckow, Curr. Op. Biotech.,

1993, 4:564-572; Griffiths and Page, 1994, Methods in Molec. biol. 75:427-440; and Merrington *et al.*, 1997, Molec. Biotech. 8(3):283-297); mammalian cells infected with adenovirus, vaccinia virus, Sindbis virus, or semliki forest virus; and mammalian cells transfected with DNA vectors for transient or constitutive (stable) expression.

5   A host cell is "transfected" by exogenous or heterologous DNA (e.g. a DNA construct) when such DNA is introduced into the cell. The transfecting DNA may or may not be integrated (covalently linked) into the host cell chromosomal DNA. In prokaryotes, yeast, and mammalian cells for example, the transfecting/transforming DNA may be maintained on an episomal element such as a

10   plasmid. With respect to eukaryotic cells, an example of a stably transfected cell is one in which the transfecting DNA has become integrated into the host cell chromosome and is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transfecting DNA.

15   Transfection methods are well known in the art (Sambrook *et al.*, 1989, *supra*; Ausubel *et al.*, 1994, *supra*).

The nucleotide sequences and polypeptides useful to practice the invention include without being limited thereto, mutants, homologs, subtypes, alleles, and the like. It is understood that generally, the sequences of the present invention

20   encode a functional protein. It will be clear to a person skilled in the art that the present invention comprises all variants, derivatives or fragments thereof, that express a functional protein.

As used herein, the designation "variant " denotes in the context of this invention a sequence, whether a nucleic acid or amino acid, that retains a biological

25   activity (either functional or structural) that is substantially similar to that of the original sequence. This variant or equivalent may be from the same or different species and may be a natural variant or be prepared synthetically. Such variants include amino acid sequences having substitutions, deletions, or additions of one or more amino acids, provided the biological activity of the protein is conserved. The

30   same applies to variants of nucleic acid sequences which can have substitutions, deletions, or additions of one or more nucleotides, provided that the biological activity of the sequence is generally maintained.

The term "derivative" is intended to include any of the above described variants when comprising additional chemical moiety not normally a part of these

35   molecules. These chemical moieties can have varying purposes including, improving

a molecule's solubility, absorption, biological half life, decreasing toxicity and eliminating or decreasing undesirable side effects. Furthermore, these moieties can be used for the purpose of labeling, binding, or they may be comprised in fusion product(s). Different moieties capable of mediating the above described effects can

5   be found in *Remington's The Science and Practice of Pharmacy* (1995). Methodologies for coupling such moieties to a molecule are well known in the art.

The term "fragment" refers to any segment of an identified DNA, RNA or amino acid sequence and/or any segment of any of the variants or derivatives described herein above.

10   The terms "variant", "derivative", and "fragment" of the present invention refer herein to proteins or nucleic acid molecules, which can be isolated/purified, synthesized chemically or produced through recombinant DNA technology. All these methods are well known in the art.

The term "APP" is an abbreviation for Amyloid-β Precursor Protein.

15   These are used interchangeably. Different forms of APPs are known in the art and are designated according to the length of the amino acid sequence, non-limiting examples include APP^{695} (Genbank accession number CAA31830), APP^{751} (Genbank accession number CAA30050) and APP^{770} (Genbank accession number CAA02049). APP^{751}, can be referred to as the intermediate form APP. For the purpose of the present

20   application the intermediate form APP is used, all the referred to amino acid residues numbers are based on the APP^{751} form.

The term "APPLP" is an abbreviation for Amyloid-β Precursor Like Protein. These are used interchangeably. APLPs are members of the amyloid precursor protein family. For the purpose of the present invention APLPs having at

25   least one caspase cleavage site are within the scope of the instant invention. Forms of APLPs having at least one caspase cleavage site are known in the art, non-limiting examples include APLP1 (Swissprot accession number P51693 and APLP2 (Swissprot accession number A49321).

The terms "epitope" and "antigenic determinant" are likewise used in

30   the conventional sense and refer to a site on an antigen that is recognized by an antibody. Neo-epitope refers to an epitope that is recognized by the antibodies described herein, this recognition is subsequent to a cleavage event on APP caused by a caspase enzyme, said epitope being substantially unrecognized in the absence of such a cleavage event.

Single letter abbreviations for amino acids are used and have their standard conventional meanings.

Specific Embodiments

5   The present invention thus concerns the development of agents capable of identifying cleavage products generated by protease proteolysis of members belonging to the amyloid precursor protein family, particularly the cleavage products generated as a result of cleavage by a caspase enzyme. Members belonging to the amyloid precursor protein family and having at least one caspase cleavage site are

10   within the scope of this invention, non-limiting examples of such members include APLPs and APPs, more particularly, APLP1 and APLP2, and APP^{695}, APP^{751} and APP^{770}, respectively.

One of the consequences of caspase cleavage of APP and APLP1 is the creation or exposing of neo-epitopes at the cleavage sites of the cleaved protein, or as

15   a result of such cleavage. Raising and isolating antibodies able to specifically bind to these neo-epitopes provides agents that selectively identify these cleavage products.

Accordingly, the present invention provides antibodies that are capable of binding specifically and selectively to an APP and APLP caspase cleaved product.

Thus, a first embodiment of the present invention is directed to an

20   antibody that is capable of binding to a neo-epitope, created or exposed following a caspase mediated cleavage of APP and APLP.

In a particular aspect of this embodiment, the caspase is selected from caspase 3, caspase 6 and caspase 8.

In another aspect of the invention, caspase 3 has more than one

25   cleavage site along the APPs and at least one along the APLPs. In a particular aspect, cleavage site along the APLP1 and APLP2 are at the amino acid residue number 620 and 732, respectively. The cleavage sites of the intermediate APP, based on the amino acid numbering of APP^{751}, are at the amino acid residue number 197, 219 and 720, these are sites designated: APP^{197}, APP^{219} and APP^{720}, respectively

30   In an aspect of this invention, there is provided an antibody capable of binding to the neo-epitope created or exposed by caspase 3 cleavage at the designated cleavage site of APP^{751} including APP^{197} APP^{219} and APP^{720}.

In a preferred aspect of this invention, there is provided an antibody capable of binding to the neo-epitope created or exposed by caspase 3 cleavage at the

35   designated cleavage site of APP^{751}, APP^{720}.

In another aspect there is provided an antibody raised with an antigenic determinant comprising a peptide of at least four amino acid residues and containing the caspase consensus sequence V/DXXD. The antibody is capable of recognizing a neo-epitope created or exposed following caspase mediated cleavage of an amyloid precursor protein family member including APLPs and APPs. Preferably the amyloid precursor protein family member is an APP including APP^{695}, APP^{751} and APP^{770}. More preferably the family member is APP^{751}.

In a preferred aspect there is provided an antibody raised with an antigenic determinant comprising a peptide sequence corresponding to the amino acid residues APP^{714} to APP^{720}, based on the APP^{751}.

In a specific aspect and for the purpose of enhancing antigenicity, the antigenic determinant further comprises an additional amino acid residue for coupling to the carrier protein KLH (Keyhole Limpet Hemocyanin). This is a method well known in the art; any other means capable of enhancing antigenicity are within the scope of this invention.

In a more specific aspect the coupling amino acid residue is at the amino terminal of the antigenic determinant.

In a most specific aspect the coupling amino acid is cysteine and the antigenic determinant comprises KLH-cysteine- APP^{714} to APP^{720}.

In another aspect of the invention the antibodies are, purified polyclonal antibodies that specifically bind to the target, the created or exposed epitope. Monoclonal antibodies raised in accordance with the antigenic determinant of the present invention for the purpose of recognizing caspase cleaved APLP and APP products, using conventional methods, are also within the scope of the present invention.

In another aspect, there is provided an antigenic determinant for immunizing a mammal in order to furnish an anti-serum containing an antibody that is capable of binding to a neo-epitope, created or exposed following caspases mediated cleavage of APLP1 and APP.

In another aspect of this embodiment the antigenic determinant comprises a peptide having at least four amino acid residues and containing the caspase consensus sequence V/DXXD.

In another aspect of this invention, there is provided a method for generating an antibody capable of specifically binding to a neo-epitope, created or exposed following a caspase mediated cleavage of APLP and APP comprising:

immunizing a mammal with a peptide having at least four amino acid residues and containing the caspase consensus sequence V/DXXD to provide antisera containing antibodies against said peptide;

collecting said antisera; and

5   selecting for an antibody specific to a caspase generated neo-epitope.

The presence of APP and amyloid β-peptide have been studied in the CSF of individuals by Carroll et al., and in multiple tissues including plasma in transgenic mice by Fukuchi et al. These references show that APP and cleaved species of APP are found in tissues other than brain. It is believed that APLP and

10   APLP caspase cleavage products can be similarly found in tissue other than brain. Thus, the presence of APLP and APP caspase cleavage products in tissues such as blood, plasma and CSF would allow easy access to a clinical specimen in which neuronal apoptosis can be detected and evaluated. Therefore, the antibodies of the present invention are useful in a diagnostic method in which APLP and APP cleaved

15   products can be detected in a clinical specimen. More specifically the antibodies of the present invention provide a diagnostic tool for the detection of at least one caspase cleaved APP product in a clinical specimen.

The antibodies of this invention, capable of specifically recognising neo-epitopes exposed following caspase mediated cleavage of APP, are useful in a

20   method for diagnosing conditions in which there is neuronal apoptosis. Examples of such conditions include neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, progressive multiple sclerosis, head trauma, prion related conditions, Creutzfeldt-Jacob disease, spongiform encephalopathy, Friedreich's ataxia, fatal familial insomnia, Pelizaeus-

25   Merzbacher disease, schizophrenia, dentatorubropallidoluysian atrophy, spinocerebellar atrophy type 3, spinal bulbar muscular atrophy, spinal cord injury, stroke and brain injury. Because caspase cleaved APP fragments can be detected in mammals using tissues other than brain, detection thereof can be used as an indicator in disease detection and disease progression. Non-limiting examples of tissues that

30   are easily accessible for use in a diagnostic method in a mammal include plasma and cerebrospinal fluid.

In a particular embodiment, the antibodies of the present invention can be used in the detection of apoptotic neuronal cells.

More particularly, by using the antibodies described herein, it is

35   possible to follow the evolution of neuronal cells undergoing apoptosis.

In an application of the present invention, there is provided a use of these antibodies in selecting test agents as modulators of apoptosis. Modulating compounds include inducers and inhibitors of apoptosis.

In an aspect of the present application, neuronal cells can be artificially induced into apoptosis and the progression of the apoptotic process followed using the antibodies of the present invention. Particularly, the antibodies can be used to study the evolution of neuronal apoptosis.

In another aspect of the present application, the progression of apoptosis can be evaluated in the presence or absence of test compounds and the results compared.

In a specific aspect of the present application, there is provided an assay system for selecting modulators of neuronal apoptosis.

In a more specific aspect of the present application, there is provided an assay for identifying inhibitors of neuronal apoptosis. Such an assay can be scaled-up to a high throughput system.

In an aspect of this application, candidate inhibitors identified using the antibodies of the present invention may be comprised in a pharmaceutical composition for treating mammals including humans in need of such treatment.

In an additional aspect of this invention, the antibodies generated to detect caspase mediated APP cleavage products can be included in a kit. Such a kit would comprise at least one antibody capable of binding to at least one neo-epitope, exposed following caspase mediated cleavage of APP. This could have applications for research or diagnostic purposes.

The invention is further illustrated in connection with the following examples.

EXAMPLE 1

Recombinant engineering

All APP clones were derived from human APP^{751}. Clone designations below are in the following format: [construct]: [vector]: [insert site/ insert release]: [sense orientation]: [identifier]. Clones were generated, as indicated, by PCR-mediated template modification and were fully sequenced before use. Clones for *in vitro* transcription/ translation include: {[wtAPP^{751}; Met^1-Asn^{751}]: [pBII KS+]: [*EcoRI / SmaI ClaI*]: [T7]: [MF-UMP#1335]};

{[ΔN-APP^{751}; Met^1-Ala^{17} Gly^{251}-Asn^{751}]: [pBII KS+]: [*Eco*RI / *Sma*I *Cla*I]: [T7]: [MF-UMP#4476]};

{[APP^{751}; D^{720}A]: [pBII KS+]: [*Eco*RI / *Sma*I *Cla*I]: [T7]: [MF-UMP#4444];

{[APP^{751}; D^{219}A, D^{720}A]: [pBII KS+]: [*Eco*RI / *Sma*I *Cla*I]: [T7]: [MF-UMP#4482]};

{[APP^{751}; D^{197}A, D^{219}A, D^{720}A]: [pBII KS+]: [*Eco*RI / *Sma*I *Cla*I]: [T7]: [MF-UMP#4540]};

{[APP^{751}-ΔC; Met^1-Asp^{720}]: [pBII KS+]: [*Eco*RI *Cla*I / *Sma*I *Cla*I]: [T7]: [MF-UMP#4446]};

{[sigΔN-APP^{751} VNLD; Met^1-Ala^{17} Gly^{251}-Asn^{751}]: [pBII KS+]: [*Bam*HI / *Cla*I]: [T7]: [MF-UMP#4476]};

{[sigΔN-APP^{751} VNLD ('Swedish'); Met^1-Ala^{17} Gly^{251}-Asn^{751}, K^{651}N, M^{652}L]: [pBII KS+]: [*Bam*HI / *Cla*I]: [T7]: [MF-UMP#4982]};

{[sigΔN-APP^{751} VNLA; Met^1-Ala^{17} Gly^{251}-Asn^{751}, K^{651}N, M^{652}L, D^{653}A]: [pBII KS+]: [*Bam*HI / *Cla*I]: [T7]: [MF-UMP#4983]}; and

{[sigΔN-APP^{751} VEHD; Met^1-Ala^{17} Gly^{251}-Asn^{751}, K^{651}E, M^{652}H]: [pBII KS+]: [*Bam*HI / *Cla*I]: [T7]: [MF-UMP#5165]}.

Mammalian expression clones include:

{[wtAPP^{751}; Met^1-Asn^{751}]: [pCEP4]: [*Not*I *Xmn*I-blunt-*Bam*HI]: [CMV]: [MF-UMP#4573]};

{[APP^{751}; D^{720}A]: [pCEP4]: [*Not*I *Xmn*I-blunt-*Bam*HI]: [CMV]: [MF-UMP#4575]};

{[APP^{751}; D^{197}A, D^{219}A, D^{720}A]: [pCEP4]: [*Not*I *Xmn*I-blunt-*Bam*HI]: [CMV]: [MF-UMP#4901]};

{[APP^{751}-ΔC; Met^1-Asp^{720}]: [pCEP4]: [*Not*I *Cla*I-blunt-*Bam*HI]: [CMV]: [MF-UMP#4577]};

{[APP^{751} VNLD; K^{651}N, M^{652}L]: [pCEP4]: [*Not*I *Xmn*I-blunt-*Bam*HI]: [CMV]: [MF-UMP#5149]}; and

{[APP^{751} VNLA; K^{651}N, M^{652}L, D^{653}A]: [pCEP4]: [*Not*I *Xmn*I-blunt-*Bam*HI]: [CMV]: [MF-UMP#5150]}.

EXAMPLE 2

Stable cell lines

To generate stable cell lines, B103 cells (a rat neuronal cell line) or K562 cells (a human erythroleukemia cell line) were transfected with the indicated

pCEP4 plasmid then selected continuously with hygromycin. Expression levels were determined during the course of each experiment by immunoblotting to verify that each cell line expressed comparable levels of their respective APP constructs. Cell line designations below are in the following format: [designation]: [expressed protein]: [transfection clone]: [selection]: [identifier]. Stable cell lines include:

{[B103-APP^{751}]: [wtAPP^{751}; Met^1-Asn^{751}]: [MF-UMP#4573]: [hygro]: [B103:4573; 97106-22]};

{[B103-APP D^{720}A]: [APP^{751}; D^{720}A]: [MF-UMP#4575]: [hygro]: [B103:4575; 97106-22]};

{[B103-APP D^{197}A, D^{219}A, D^{720}A]: [APP^{751}; D^{197}A, D^{219}A, D^{720}A]: [MF-UMP#4901]: [hygro]: [B103:4901; 97106-43]};

{[B103-APP^{751}-ΔC]: [APP^{751}-ΔC; Met^1-Asp^{720}]: [MF-UMP#4577]: [hygro]: [B103:4577; 97106-22]};

{[K562-APP^{751}]: [wtAPP^{751}; Met^1-Asn^{751}]: [MF-UMP#4573]: [hygro]: [K562:4573; 97106-16b]};

{[K562-APP^{751} VNLD ('Swedish')]: [APP^{751}; K^{651}N, M^{652}L]: [MF-UMP#5149]: [hygro]: [K562:5149; 97106-64]}; and

{[K562-APP^{751} VNLA]: [APP^{751}; K^{651}N, M^{652}L, D^{653}A]: [MF-UMP#5150]: [hygro]: [K562:5150; 97106-64]};

EXAMPLE 3

Generation of antibodies that specifically recognize a caspase-generated APP neo-epitope (αΔC^{Csp}-APP)

Two rabbits were immunized with the peptide that includes the seven amino acids in APP that precede the caspase cleavage site at aspartic acid 720 (N terminal APP^{714} to C terminal APP^{720}, inclusive) as well as an amino-terminal cysteine residue to allow coupling to KLH. The rabbits were boosted three times with the immunizing peptide over a 10 week period. At 10 weeks, the titers against the immunizing peptide (determined by ELISA and defined as the maximal dilution giving a positive response, arbitrarily set at OD$_{450}$ = 0.2 using the chromogenic substrate for horse radish peroxidase ABTS) were >1:204,800 and 1:12,520. The antisera were pooled and affinity purified by three successive chromatographic steps: (1) the antisera was depleted of immunoglobulins recognizing intact APP by adsorption to a bridging peptide that spans the caspase cleavage site (N terminal APP^{713} to C terminal APP^{726}, inclusive) immobilized on Sepharose 4B (Pharmacia)

by cyanogen bromide activation; (2) the flow through from step (1) was applied to a Sepharose 4B column containing the immunizing peptide (immobilized to the support as described in (1)); the column was washed and the specific antibodies were eluted via a pH gradient and placed in a borate buffer (0.125 M borate); (3) the eluant from the second step was absorbed against the immobilized bridging peptide as described in (1) and the flow through was collected. The ELISA titre for this antibody preparation was <1:142,000 (<5 ng/ml) against the immunizing peptide that corresponds to the caspase-generated neo-epitope, versus >1:71 (>10 μg/ml) for the bridging peptide which corresponds to intact APP.

EXAMPLE 4

Immunocytochemical analysis of the caspase cleaved fragment of APP in apoptotic NT2 cells

Subconfluent NT2 cells were incubated with 1 μg/ml campthothecin in the presence or absence of 50 μM Z-VAD(OMe)-CH$_2$F for 4 hours at 37°C with 5% CO$_2$. The final concentration of DMSO used to dilute the camptothecin and Z-VAD(OMe)-CH$_2$F was 0.3% (v/v). As a control, cells were incubated with 0.3% (v/v) DMSO alone. The cells were detached from the plate with Cell Dissociation Solution (Sigma) and pooled with the culture media (to retain floating apoptotic cells).

Following centrifugation, the cells were fixed in 10% neutral buffered formalin for 10 min at room temperature, washed twice in phosphate-buffered saline, and then centrifuged using a Cytospin centrifuge onto poly-L-lysine-coated glass slides (Sigma) and dried overnight. The cells were then co-stained for TUNEL and either $\alpha\Delta C^{Csp}$-APP at a dilution of 1/4000 or αCsp-3(MF397) at a dilution of 1/3000, essentially as described previously (Black et al., 1998) except that the proteinase K treatment was omitted.

EXAMPLE 5

in vivo Models of acute brain injury

All animal procedures conformed to the *Guide for the Care and Use of Experimental Animals* (Canadian Council on Animal Care, 1993) endorsed by the Medical Research Council of Canada.

For kainic acid treatment adult male mice (C57/B6xXJL; 25-30 g; Charles River, Montréal) were injected with an i.p. bolus of 35 mg/kg kainic acid. After 48 hr, the animals were euthanized with an overdose of pentobarbital (100 mg/kg i.p.). Brains were harvested, sectioned and analyzed as described below.

For transient forebrain ischemia, adult male Wistar rats (250-275 g; Charles River, Montréal) were used for all of the experiments. Transient forebrain ischemia was produced using published modifications (Pulsinelli and Buchan, 1988) of the four-vessel occlusion (4-VO) method(Pulsinelli et al., 1982). Animals that convulsed during 4-VO, or following reperfusion, along with those that did not fully develop loss of righting reflex during ischemia were omitted from the study. In the case of sham treated animals, the carotids were exposed but not occluded.

EXAMPLE 6

TUNEL and fluorescence immunohistochemistry
of rodent and human brain

Fresh frozen brain sections (12 μm thick) were pre-treated with PBS containing 0.1% (v/v) Triton X-100. The sections were then incubated with a primary antisera that selectively recognizes the active form of caspase-3 (αCsp-3(MF397); a rabbit polyclonal antisera raised against (p17/p12)$_2$ caspase 3; used at 1/2000 dilution, or purified immunoglobulins that recognize the caspase-generated ΔC-APP neo-epitope ($\alpha\Delta C^{Csp}$-APP (see above) used at 1/2000 dilution) for 48 hours at 4°C. Following 3 washes with PBS (5 min each), the sections were incubated with CY^3 labeled-donkey anti-rabbit IgG (1/500, Amersham) for two hours at room temperature to visualize active caspase-3 or ΔC-APP immunoreactivity. The DNA fragmentation characteristic of apoptosis was detected by TUNEL as described previously (Xu et al., 1997).

EXAMPLE 7

DNA laddering

Extraction of DNA to visualize olignucleosomal fragmentation was performed as follows. Subconfluent NT2 cells were incubated with 1 μg/ml campthothecin in the presence or absence of 33.3 μM Z-VAD(OMe)-CH2F for 6 hours at 37°C with 5% CO_2. As a control, cells were incubated with vehicle (0.2 % (v/v) DMSO) alone. Cells were removed from the dish by scraping, pooled with the

culture medium, and pelleted at 10,000 x g for 10 min. Cell pellets (2 x 10^5
cells/pellet) were resuspended in 0.5 ml of 0.6% (w/v) SDS, 10 mM EDTA (pH 7.5)
followed by the addition of NaCl to a final concentration of 1M. After overnight
incubation at 4°C, the supernatants were clarified by centrifugation at 14,000 x g for

5   20 min at 4°C and treated with 7 μg/ml DNase-free RNase for 45 min at 37°C. The
DNA was extracted once with phenol/ chloroform/ isoamyl alcohol (25:24:1, v/v/v),
isopropanol precipitated, resuspended in loading buffer (30% (v/v) glycerol, 10 mM
EDTA pH 7.5, 0.05% (w/v) bromophenol blue), and analysed by electrophoresis on a
1.2% agarose gel in 1 x TAE (40 mM TrisAcetate, 1 mM EDTA pH 8.3). Bands were

10   visualized following ethidium bromide staining.

EXAMPLE 8

Other procedures

        Recombinant human caspases were generated as described previously

15   (Rotonda et al., 1996; Thornberry et al., 1997) and *in vitro* cleavage assays were
performed in buffer composed of 50 mM Hepes/KOH (pH 7.0), 10% (w/v) sucrose, 2
mM EDTA, 0.1% (w/v) CHAPS, 5 mM dithiothreitol. Peptide aldehydes and
fluorogenic aminomethylcoumarins were prepared by solid-phase synthesis essentially
as described before(Rano et al., 1997). Z-VAD(OMe)-CH$_2$F, a pan-caspase inhibitor

20   (Garcia-Calvo et al., 1998), was purchased from Enzyme Systems Products. NT2
cells (human neuronal precursor) and B103 cells (rat neuronal) were maintained in
DMEM containing antibiotics and 10% (v/v) fetal bovine serum (FBS). Retinoic
acid-differentiated NT2 neurons (hNT) were maintained in neuron conditioned
medium (Stratagene) except during the course of serum-deprival experiments when

25   they were cultured in DMEM (-/+ 10% (v/v) FBS). K562 cells (human
erythroleukemia) and Jurkat cells (human T- lymphoid) were maintained in RPMI-
1640 containing antibiotics and 10% (v/v) FBS. Apoptosis was induced by CD95
(Fas/APO-1) ligation using 1 μg/ml anti-Fas antibodies (Jurkats only), with 1 μM
staurosporine (all cell lines) or with 1 μg/ml camptothecin (all cell lines) as indicated.

30   Cell extracts were prepared as described previously (Nicholson et al., 1995).

RESULTS

NEURONAL NT2 CELLS PRODUCE ELEVATED LEVELS OF AMYLOID-ß
PEPTIDES DURING APOPTOSIS WHICH IS ATTENUATED BY CASPASE
INHIBITION

5   Primary human neurons undergoing apoptosis have been shown to
increase the rate of amyloid-ß peptide formation by 3- to 4-fold(LeBlanc, 1995). It
was therefore determined whether this could be reproduced in a cultured human
neuronal cell line and whether apoptosis inhibition prevented Aß production.
Retinoid-differentiated human neuronal NT2 cells (hNT) induced to undergo

10   apoptosis by serum deprivation had a substantially elevated rate of Aß peptide
formation (about 4-fold) (Fig. 1A) and this apoptosis-related increase of Aß formation
was attenuated by a non-selective irreversible caspase inhibitor. The rate of Aß
production in the presence of the caspase inhibitor was restored to the steady-state
levels found in non-apoptotic hNT cells but it was not entirely abolished, indicating

15   that apoptosis enhances the pathway leading to Aß generation, but is not obligatory
for it to occur. Acute apoptotic induction of the neuronal precursor cell line NT2 with
camptothecin (Fig 1B) resulted in oligonucleosomal DNA fragmentation and
concomitant maturation of procaspase-3, cleavage of caspase-3 substrates (such as
PARP; poly(ADP-ribose) polymerase) and a ca. 3.5 kDa reduction in the mass of

20   endogenous APP (lanes 2). The degree of proteolysis of all three polypeptides was
approximately the same (procaspase-3 = 37 ± 4%; PARP = 66 ± 9%; APP = 44 ± 4%;
$n=5$) and in each case was abolished by co-incubation with a caspase inhibitor (lanes
3). This indicates that APP is processed by a caspase-dependent event during
apoptosis and raises the possibility that caspase cleavage directly contributes to the

25   higher propensity for Aß peptide formation observed in apoptotic cells.

CASPASE-3 DIRECTLY AND EFFICIENTLY CLEAVES THE AMYLOID-ß PRECURSOR PROTEIN

Owing to the increase in APP proteolysis and Aß peptide formation that accompanies neuronal apoptosis, we sought to determine whether any of the

5   biochemical components of the cell death pathway might contribute to this process. Apoptosis itself is dependent on a family of cysteine proteases, the caspases, which manifest the apoptotic phenotype by cleaving a discrete subset of cellular polypeptides at Asp-x bonds (Alnemri et al., 1996; Nicholson and Thornberry, 1997; Thornberry et al., 1997). The core substrate recognition motif for caspases is comprised of a

10   tetrapeptide, N-terminal to the scissile bond (P_4-P_1), with an Asp in P_1 being an absolute requirement for cleavage and the P_4 residue being the major specificity determinant. The caspases normally exist as dormant proenzymes in healthy cells but are proteolytically converted to active proteases in response to diverse apoptotic stimuli. The potential direct role of caspases in APP processing was initially tested by

15   combining [^{35}S]-labeled APP with extracts from healthy versus apoptotic Jurkat cells (Fig. 1C). Jurkat cells were initially chosen because they contain multiple caspase family members (caspases-2, -3, -4, -6, -7, -8 and -9) that are readily activated during apoptosis (unpublished). The 120 kDa APP was rapidly processed to a triad of smaller polypeptides of approximately 85-90 kDa. These polypeptides corresponded

20   in mass to APP processing intermediates observed in primary human neurons deprived of trophic support (LeBlanc, 1995; LeBlanc et al., 1996) or in a human neuronal cell line during H_2O_2-induced apoptosis (Zhang et al., 1997), suggesting that the *in vitro* proteolysis of APP by cell extracts may represent that which occurs in neuronal cells. Similar APP cleavage products were also generated by apoptotic

25   extracts from other cell types, including NT2 human neuronal precursor cells and B103 neuroblastoma cells (not shown). The single APP cleavage event observed in intact NT2 cells during apoptosis (Fig. 1B) suggests that the transmembrane oritentation of APP restricts the accessibility of the cleavage sites to proteolysis and that one of the three cleavage sites might be more vulnerable *in vivo*. A protease

30   inhibitor profile indicated that all three cleavage products arose as a consequence of an E64-insensitive cysteine protease, a signature for caspases (not shown). This was confirmed by the ability of caspase-specific peptide-aldehydes to block APP processing to the 85-90 kDa triad (Fig. 1D). The caspase inhibitor, Ac-DEVD-CHO, was a potent inhibitor of APP processing (IC_{50} = 1 nM) whereas both Ac-IETD-CHO

35   and Ac-YVAD-CHO were substantially less effective. Since Jurkat cells contain

members of all three caspase subgroups, these results indicate that the group II
caspases, which include caspase-3 (a key mediator of neuronal apoptosis), primarily
account for the APP cleavage observed in apoptotic cells. Identical results were
obtained in neuronal precursor NT2 cells; not surprisingly, since caspase-3 is the

5   major caspase in this cell line ([caspase-3] ~ 55 pM; all other caspases < 14 pM;
unpublished). Collectively, these results indicate that caspase-3 predominantly
accounts for APP proteolysis during apoptosis. In support of this, the proteolytic
cleavage of the 120 kDa APP to the 85-90 kDa triad could also be reproduced with
recombinant caspase-3 (Fig. 1E) and an analysis of cleavage kinetics indicates that

10   APP proteolysis by caspase-3 occurs with relevent kinetic properties (k_{cat}/K_m = 5.14 x
10^5 $M^{-1}s^{-1}$) which are similar to validated substrates for caspase-3, such as poly(ADP-
ribose) polymerase (k_{cat}/K_m = 15.6 x 10^5 $M^{-1}s^{-1}$ in the same experiment; not shown).
Of the human caspases tested (caspases-1 to 10 and granzyme B; each examined at
equimolar concentrations under identical conditions), caspase-3 was the most efficient

15   enzyme to cleave APP, although minor cleavage also occurred with caspases-6 and -8
(not shown).

CASPASE-3 LEVELS ARE ELEVATED IN DYING NEURONS IN HUMAN ALZHEIMER'S BRAIN

20   If caspase-3 is involved in APP processing and neuronal apoptosis, it
should be present at the sites of Aß production and neurodegeneration. Caspase-3
(CPP32, apopain, Yama)(Fernandes-Alnemri et al., 1994; Nicholson et al., 1995;
Tewari et al., 1995) is one of the key effector caspases in mammalian cell suicide. Its
potential importance in neuronal cell death in particular is underscored by the

25   phenotype of caspase-3-deficient mice which have a massive defect in neuronal
apoptosis during development, leading to brain enlargement (2 to 3-fold),
supernumerary cell masses and duplicated brain structures (Kuida et al., 1996). In
Alzheimer's patients, we found an elevation of caspase-3 immunoreactivity in dying
pyramidal neurons of the hippocampus, including the CA3 region, one of the initial

30   sites of neuronal loss in Alzheimer's disease (Fig. 2). This circumstantial evidence
suggests that caspase-3 might mediate or contribute to the death of these cells during
neurodegeneration and that caspase-3-mediated proteolysis of APP may accompany
this process as a component of human Alzheimer's disease pathogenesis.

IDENTIFICATION OF THREE PRINCIPLE SITES OF CASPASE-MEDIATED APP PROTEOLYSIS

The sites of caspase proteolysis within the 120 kDa APP polypeptide were determined. This was initially facilitated by the unusual disposition of Cys

5   residues at the amino-terminus of APP. Treatment of $[^{35}S]$Cys-labeled APP with recombinant caspase-3 generated two small $[^{35}S]$Cys-containing fragments (23 and 25 kDa) and a larger polypeptide (85 kDa), indicating that two of the caspase cleavage sites were within the first N-terminal 220 amino acids of APP and a third site was present at about residue 700 (not shown). The position of the latter site was further

10   refined by cleaving an APP construct lacking the N-terminal 220 amino acids (ΔN-APP). Within each of the three regions predicted to contain the caspase cleavage sites by analysis of $[^{35}S]$Cys APP and $[^{35}S]$Met APP fragment sizes, excellent caspase consensus sites (Thornberry et al., 1997) were present at: APP^{194} to APP^{197} , APP^{216} to APP^{219} and APP^{717} to APP^{720} , inclusive, these sites are designated

15   $(P_4)DNVD^{197}(P_1)/S$, $(P_4)DYAD^{219}(P_1)/G$ and $(P_4)VEVD^{720}(P_1)/A$, respectively (Fig. 3B). That these were the authentic sites of caspase proteolysis was confirmed by site-directed mutagenesis of the essential P_1 Asp of each site (Fig. 3A). The APP triple mutant (APP $D^{197}A$, $D^{219}A$, $D^{720}A$), for example, could not be cleaved by recombinant caspases (lanes 7&8), nor by extracts from apoptotic cells (not shown).

20   (Equivalent results were observed with the small molecular mass counterparts of the fragments described in Fig. 3A (not shown).)

CASPASE-MEDIATED APP PROTEOLYSIS OCCURS IN INTACT CELLS, PREDOMINANTLY AT ASP^{720}, AND IS ELEVATED DURING APOPTOSIS

25   Cleavage at the sites described above was next tested in intact cells by stable transfection of B103 neuronal cells with APP containing either wild-type or P_1 Asp-to-Ala mutations. (B103 cells were chosen owing to their very low levels on endogenous APP, which facilitates the analysis of transfected site-directed mutants.) When B103 cells harboring stably-transfected wild-type APP were induced to

30   undergo apoptosis, the caspase-mediated cleavage at the carboxy-terminal $(P_4)VEVD^{720}(P_1)/A$ site was observed (Fig. 4A lanes 1 & 2). This cleavage event was entirely eliminated by the triple mutant (not shown) or by a $D^{720}A$ point mutant (lanes 5 & 6), substantiating the direct involvement of caspases in APP processing in intact neuronal cells. Non-apoptotic cells also cleaved APP at the carboxy-terminus at ca.

15% of the rate occurring in apoptotic cells (lane 1 vs. 2) and this too was eliminated by mutation of the P_1 Asp (D^{720}A) that is required for caspase recognition (lane 5). Cleavage at the VEVD^{720}A site is consistent with the approx. 3.5 kDa reduction in APP observed during apoptosis in NT2 cells (see Fig 1B, lane 2) and appears, at least

5   with the cell lines tested, to be the predominant site of caspase proteolysis in intact cells (although APP fragments corresponding to cleavage at the two upstream sites have been observed by others (Zhang et al., 1997)). Positive identification of this C-terminal cleavage event was confirmed using a neo-epitope antibody that we generated for immunohistochemical detection of caspase-generated ΔC-APP *in situ*.

10   The antisera was raised against a synthetic peptide corresponding to the *nouveau* C-terminus of ΔC-APP, [KLH-cysteine- APP^{714} to APP ^{720}], then purified by immunoadsorption to the same peptide and two cycles of immunodepletion with a bridging peptide corresponding to intact APP straddling the cleavage site comprising the amino acid residues APP^{714} to APP^{726}, inclusive. This antibody, designated αΔ

15   C^{Csp}-APP, was confirmed to be highly specific for the caspase-generated neo-epitope in APP by three criteria: a) its titre by ELISA was > 2000-fold selective for the immunizing peptide (corresponding to ΔC-APP) versus the bridging peptide harboring the same sequence (which corresponds to intact APP) (see Methods), b) the antibody could immunoprecipitate biosynthetic [^{35}S]APP that was truncated at the

20   D^{720} caspase site but not intact APP (Fig. 4B), and c) the antibody could not immunoprecipitate intact APP from healthy NT2 cells, but following apoptosis induction was able to immunoprecipitate the ΔC-APP cleavage product (Fig 4C). In the latter case, formation of immunoprecipitable ΔC-APP in NT2 cells was abolished by the presence of a caspase inhibitor (lane 3 vs. 2). Comparable results were

25   obtained by immunohistochemical staining of NT2 cells (Fig. 4D-I). NT2 cells induced to undergo apoptosis with camptothecin became TUNEL positive with concomitant positive staining for active caspase-3 (panel E) and for the caspase-generated APP neo-epitope (panel H). All three of these apoptosis-related events were abolished by the presence of a caspase inhibitor (panels F and I). Collectively,

30   these experiments demonstrate that caspase-mediated proteolysis of APP at the C-terminal VEVD^{720}A site occurs during apoptosis in intact cells. Furthermore, the ability of the αΔC^{Csp}-APP neo-epitope antibody to recognize the terminus generated by caspase proteolysis *in situ* makes it a useful tool for following this cleavage event *in vivo*.

35

CASPASE-MEDIATED APP PROTEOLYSIS OCCURS IN HIPPOCAMPAL
NEURONS *IN VIVO* DURING ACUTE BRAIN INJURY

The early loss of hippocampal neurons is a prominent feature in
Alzheimer's disease pathogenesis. We therefore wanted to determine whether
5   caspase-mediated APP proteolysis could be detected *in vivo* during hippocampal cell
death. Two experimental models of acute brain injury, both resulting in the apoptotic
loss of hippocampal neurons, were used to examine these events: a) kainic acid
(excitotoxic)-induced seizures where CA3 neurons predominantly die, and b) global
cerebral ischemia in which CA1 neurons perish. These models have an additional
10 advantage in that acute brain injury is an established risk factor for Alzheimer's
disease susceptibility and may therefore shed light on the molecular basis for this
increased vulnerability. In the excitotoxic model of neuronal injury (Fig. 5A-F),
caspase-mediated APP proteolysis, measured by $\alpha\Delta C^{Csp}$-APP immunoreactivity, was
not detectable in the CA3 region of the hippocampus of control mice (panel A)
15 whereas intense $\alpha\Delta C^{Csp}$-APP immunoreactivity was observed in CA3 neurons of
SV129 mice 48 hr after administration of kainic acid (25 mg/kg, s.c.) (panel B).
Consistent with the generation of this fragment by a caspase-3 dependent mechanism,
a pronounced elevation of active caspase-3 immunoreactivity was observed in the
CA3 region of adjacent hippocampal tissue sections from animals injected with kainic
20 acid (panel D) but not vehicle (panel C). TUNEL labeling confirmed that the CA3
neurons which displayed $\alpha\Delta C^{Csp}$-APP and active caspase-3 immunoreactivity were
apoptotic (panel F). To further establish that caspase-mediated APP cleavage occurs
in vivo, we determined whether the apoptotic death of CA1 neurons produced by a
brief episode of global ischemia was also accompanied by increased production of
25 ΔC-APP (Fig. 5G-L). Basal $\alpha\Delta C^{Csp}$-APP immunoreactivity was not observed in the
CA1 region of rats subjected to a sham surgical procedure (panel G); however, $\alpha\Delta$
C^{Csp}-APP immunoreactivity was clearly visible in vulnerable CA1 neurons 24 hr after
transient global ischemia produced by 12 min of four-vessel occlusion (panel H). By
comparison to the 24 hr time point, $\alpha\Delta C^{Csp}$-APP immunolabeling appeared to be
30 more intense at 48 hr (panel I) whereas at 72 hr., immunoreactivity was only seen in a
few remaining CA1 neurons (panel J). The time courses for generation of active
caspase-3 and $\alpha\Delta C^{Csp}$-APP immunoreactivity were similar in CA1 neurons following
this short period of global ischemia (data not shown). In agreement with previous
findings, which indicate that CA1 neurons undergo apoptosis following transient
35 global ischemia, TUNEL labeling was elevated in CA1 neurons (panel K). Under

high magnification, $\alpha\Delta C^{Csp}$-APP immunoreactivity was apparent in dendritic

processes as well as the cell body of dying CA1 neurons (panel L). Taken together,

these results indicate that neuronal apoptosis in the rodent hippocampus, produced by

either excitotoxic or ischemic injury, is associated with an elevation in ΔC-APP

5   formation. Consistent with our *in vitro* findings which indicate that the ΔC-APP

fragment is derived by caspase-mediated proteolysis of APP, increased production of

ΔC-APP *in vivo* accompanied the generation of active caspase-3 in apoptotic neurons.

Caspase-mediated alteration of APP processing after acute brain injury may in part

account for the increased susceptiblity to Alzheimer's disease in human patients as

10   well.

CASPASE-CLEAVED APP CO-LOCALIZES WITH SENILE PLAQUES IN
HUMAN ALZHEIMER'S BRAIN

The relevance of caspase-mediated cleavage of APP for senile plaque

15   formation was assessed in human patients that were diagnosed with late stage

Alzheimer's disease (Fig. 6). Senile plaques and $\alpha\Delta C^{Csp}$-APP immunoreactivity

were rarely observed in age-matched control patients that did not suffer from

Alzheimer's disease. In contrast, numerous amyloid-β-positive plaques were

observed in hippocampal sections from patients that suffered from Alzheimer's

20   disease (panel B) and $\alpha\Delta C^{Csp}$-APP immunoreactivity was frequently observed (>90%)

in the senile plaques (panel A). These deposits had a punctate appearance and were

localized within the senile plaques (panel C); however, it was not possible on

morphological grounds to define the cellular phenotype in which the $\alpha\Delta C^{Csp}$-APP

immunoreactivity was located. These findings are consistent with the hypothesis that

25   caspase-generated ΔC-APP contributes to plaque formation.

CASPASE-MEDIATED PROTEOLYSIS OF APP INCREASES THE RATE OF
AMYLOID-ß PEPTIDE FORMATION IN NEURONAL CELLS

Since APP is cleaved by caspases during neuronal injury *in vivo*, we

30   next determined whether this had an effect on the endogenous APP processing

pathway leading to Aß peptide formation. This was tested in stably-transfected B103

cells harboring the C-terminal-truncated APP construct ($\alpha\Delta$-APP) corresponding to

the product of caspase proteolysis at the D^{720} site (Fig. 7). From these cells, Aß

peptide production was elevated 5-fold compared to cells that were stably transfected

and expressing identical levels of full length APP (column 2 vs 1 at 20h, 4 vs 3 at
48h). One possible explanation for this effect, given that caspase proteolysis does not
directly account for Aß excision from APP, is that caspase proteolysis adulterates the
normal endogenous metabolism of APP and that the truncated molecule has a higher
5   propensity for metabolism by an amyloidogenic pathway, perhaps by dissociation of
key interaction domains contained within the C-terminus of the intact APP
polypeptide (summarized in Fig. 3B). It has recently been demonstrated, for example,
that the Shc-like PTB domain of X11 interacts with the NPTY motif at the C-terminus
of APP and reduces Aß peptide formation by binding to and slowing APP processing
10   (Borg et al., 1998). It is likely that removal of this and other key components of the
C-terminus of APP biases its route of cellular degradation along an amyloidogenic
pathway (Russo et al., 1998).

THE 'SWEDISH' FAMILIAL MUTATION OF APP MARKEDLY IMPROVES AN
15   ## OTHERWISE POOR CASPASE CONSENSUS SITE IN THE ß-SECRETASE
REGION

In addition to the three caspase cleavage sites contained within the
APP polypeptide, with the dominant processing site being at Asp^{720} (Fig. 3B), at least
one documented familial Alzheimer's disease (FAD) mutation introduces an
20   additional caspase-susceptibility site to the molecule. The 'Swedish' mutation of APP
is associated with an inherited form of Alzheimer's disease with an earlier onset and
elevated production of 4 kDa amyloidogenic Aß peptides (Cai et al., 1993; Citron et
al., 1992; Haass et al., 1995; Holcomb et al., 1998). The mutation changes the
$VKMD^{653}$ sequence (amino acid residues corresponding to APP^{650} to APP^{653}) at the ß-
25   secretase site to $VNLD^{653}$, a motif with substantially improved caspase recognition
elements. In particular, the 'Swedish' mutation generates a site that would be
preferentially recognized by group III caspases, especially caspase-6, as predicted by
our positional-scanning combinatorial substrate library (Thornberry et al., 1997). To
test this possibility, fluorogenic tetrapeptides corresponding to wild type and
30   'Swedish' sequences were synthesized and tested for their ability to be cleaved by
recombinant caspase-6. The in vitro cleavage of VNLD-AMC (Swedish) was
markedly higher than the VKMD-AMC (wild type) fluorogenic peptide, although
lower than a peptide containing the optimal caspase-6 primary recognition sequence
predicted by the combinatorial library, VEHD-AMC (Fig. 8A). A differential also
35   occurred in the context of the full-length APP polypeptide with cytosolic extracts

from apoptotic cells as well as with recombinant caspase-6 (Fig. 8B). In both cases, the 'Swedish' mutation accelerated the rate of proteolysis at the ß secretase site (columns 3 & 4) and this cleavage was entirely abolished by mutation of the P_1 Asp ($D^{653}A$) that is essential for caspase recognition (columns 5 & 6). The likelihood that

5   the ß cleavage activity observed in apoptotic cellular extracts is attributable to caspase-6 was supported by its inhibitor profile (potency of Ac-IETD-CHO > Ac-DEVD-CHO > Ac-YVAD-CHO) which is consistant with a group-III caspase (which includes caspase-6) being responsible (not shown). A measurement of the rate of caspase-6-mediated proteolysis of the ß-secretase site (Fig. 8C) indicated that the

10   'Swedish' mutation improved it as a caspase-6 site within the APP polypeptide by 3-fold ($k_{cat}/K_m = 0.8 \times 10^4 \ M^{-1}s^{-1}$ for wild type APP versus $2.2 \times 10^4 \ M^{-1}s^{-1}$ for APP containing the 'Swedish' mutation). Neither the wild-type APP nor APP containing the 'Swedish' mutation could be cleaved at this site by other caspases, such as caspase-1, -3, -7, and -8, or by granzyme B (not shown), indicating that this event may

15   be restricted to caspase-6.

ENHANCED AMYLOID-ß PEPTIDE FORMATION OCCURS IN CELLS HARBORING THE 'SWEDISH' MUTATION OF APP AND IS DEPENDENT ON CASPASE PROTEOLYSIS AT THE ß-SECRETASE SITE

20   The consequences of the 'Swedish' mutation in stably-transfected K562 cells was consistent with that reported previously (Citron et al., 1992; Citron et al., 1995), including a substantial elevation in the cellular production of amyloid-ß peptides (Fig. 8D). The formation of the amyloidogenic 4 kDa Aß peptide, but not

25   the 3 kDa Aß peptide, was dramatically reduced in K562 cells containing 'Swedish' APP where the P_1 Asp necessary for caspase proteolysis was eliminated from the ß-secretase region ($D^{653}A$ lanes 3 vs 2, 6 vs 5). An Aß (1-40) sandwich Elisa produced identical results (substantially elevated Aß production in cells harboring the 'Swedish' mutation (65 ± 4 arbitrary units versus undetectable levels in cells harboring wildtype

30   APP) and attenuation by the P_1 $D^{653}A$ mutation (16 ± 4 arbitrary units)). Similar results were found in B103 neuronal cells expressing the same constructs (intracellular 4 kDa Aß peptide formation was ablated, as observed in K562 cells, but alternative cleavage to produce a <4 kDa extra-cellular Aß peptide occurred in $D^{653}A$ cell culture supernatants; probably at Phe^{656} as reported before (Citron et al., 1995)

35   (not shown)). Taken together, these findings directly implicate a caspase in the

enhanced Aß production that occurs in cells harboring the 'Swedish' mutation of
APP. Other evidence also supports the possibility that caspases (specifically caspase-
6) may contribute to ß-secretase activity, particularly in the 'Swedish' Alzheimer's
disease mutation. First, although there is heterogeneity in the amino-terminus of Aß

5   peptides, some of the peptides generated correspond to cleavage at the V (K or N)(M
or L) D^{653}/A^{654} caspase-6 site(Wang et al., 1996). Second, an analysis of the amino
acid residues within APP that constitute the minimum recognition region for the
'major protease' that accounts for ß-secretase activity (determined to be Val^{650} to
Ala^{654}) corresponds exactly with the P_4-P_1' caspase-6 recognition site (Citron et al.,

10   1995). Finally, caspase-6 is present in the hippocampus and frontal cortex of human
Alzheimer's brain samples (as well as age-matched controls) at levels that can be
readily detected by immuno-blotting (not shown). Collectively, these findings support
a role for caspase-6 in the cleavage of APP at the ß-secretase region and suggest a
caspase-assisted mechanism that accounts for the elevated Aß peptide formation in

15   patients with the 'Swedish' familial mutation.
              Caspases contribute to the complex proteolytic processing of the
amyloid-ß precursor protein and appear to contribute to amyloidogenic Aß peptide
formation by at least two distinct mechanisms. The cleavage of APP at endogenous
caspase consensus sites ((P_4)$DNVD^{197}$(P_1)/S, (P_4)$DYAD^{219}$(P_1)/G and,

20   predominantly, (P_4)$VEVD^{720}$(P_1)/A) probably corrupts the normal intracellular
processing of APP that would otherwise preclude it from Aß peptide formation or
accumulation. A 'vicious cycle', occuring over a protracted period of time, may
therefore proceed via the following steps in susceptible neurons: a) caspase-3-
mediated truncation of APP at the C-terminus, b) adulteration of normal APP

25   processing with shunting of the residual polypeptide towards an amyloidogenic
pathway, c) generation of elevated levels of cytotoxic Aß peptide species, resulting in
Aß-induced neuronal stress, d) progressive up-regulation and/or activation of
endogenous caspases, and e) exacerbated APP proteolysis. Furthermore, elevation in
APP levels (which is susceptible to caspase cleavage) has been reported in dying

30   motor neurons (Barnes et al., 1998), potentially feeding more APP into the cycle. An
apoptotic initiation event is not necessarily required for this cycle to progress since
caspase proenzymes are catalytically competent, although at low levels compared to
their mature counterparts (Muzio et al., 1998; Srinivasula et al., 1998; Yang et al.,
1998). A second mechanism involves caspase-6-mediated proteolysis in the ß-

35   secretase region; a process that is accelerated by residue changes at this site, such as

the 'Swedish' dipeptidyl mutation, that improve its susceptibility to this enzyme. In addition to APP cleavage, caspases have recently been implicated in alternative presenilin proteolysis (Kim et al., 1997; Vito et al., 1997). Caspases may thus play an important role in both the generation of neurotoxic Aß peptides as well as in the

5   ultimate death of neurons by apoptosis in Alzheimer's disease.

References

Alnemri, E. S., Livingston, D. J., Nicholson, D. W., Salvesen, G., Thornberry, N. A., Wong, W. W., and Yuan, J. (1996). Human ICE/CED-3 protease nomenclature

10   [letter]. Cell 87, 171.

Barnes, N. Y., Li, L., Yoshikawa, K., Schwartz, L. M., Oppenheim, R. W., and Milligan, C. E. (1998). Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons. J Neurosci 18, 5869-80.

15

Black, S. C., Huang, J. Q., Rezaiefar, P., Radinovic, S., Eberhart, A., Nicholson, D. W., and Rodger, I. W. (1998). Co-localization of the cysteine protease caspase-3 with apoptotic myocytes after in vivo myocardial ischemia and reperfusion in the rat. J Mol Cell Cardiol 30, 733-42.

20

Borg, J. P., Yang, Y., De Taddeo-Borg, M., Margolis, B., and Turner, R. S. (1998). The X11alpha protein slows cellular amyloid precursor protein processing and reduces Abeta40 and Abeta42 secretion. J Biol Chem 273, 14761-6.

25   Cai, X. D., Golde, T. E., and Younkin, S. G. (1993). Release of excess amyloid beta protein from a mutant amyloid beta protein precursor. Science 259, 514-6.

Carroll, R.T, Lust, M.R., Kim, K.S., Doyle, P.D. and Emmering, M.R. (1995). An age-related correlation between levels of beta-amyloid precursor protein and beta-

30   amyloid in human cerebrospinal fluid. Bichem Biophys Res Commun 210,345-349

Citron, M., Oltersdorf, T., Haass, C., McConlogue, L., Hung, A. Y., Seubert, P., Vigo-Pelfrey, C., Lieberburg, I., and Selkoe, D. J. (1992). Mutation of the beta-amyloid precursor protein in familial Alzheimer's disease increases beta-protein

35   production. Nature 360, 672-4.

Citron, M., Teplow, D. B., and Selkoe, D. J. (1995). Generation of amyloid beta protein from its precursor is sequence specific. Neuron *14*, 661-70.

5   Cotman, C. W., and Su, J. H. (1996). Mechanisms of neuronal death in Alzheimer's disease. Brain Pathol *6*, 493-506.

Fernandes-Alnemri, T., Litwack, G., and Alnemri, E. S. (1994). CPP32, a novel human apoptotic protein with homology to Caenorhabditis elegans cell death protein
10 Ced-3 and mammalian interleukin-1 beta- converting enzyme. J Biol Chem *269*, 30761-4.

Fukuchi, K., Ho, L., Younkin, S.G., Kunkel, D.D., Ogburn, C.E., LeBoeuf, R.C., Furlong, C.E., Deeb, S.S., Nochin, D., Wegiel, J., Wisniewski, H.M. (1996). High
15 levels of circulating beta-amyloid peptide do not cause cerebral beta-amylodosis in trangenic mice. Am J Pathol *149*, 219-227

Forloni, G., Bugiani, O., Tagliavini, F., and Salmona, M. (1996). Apoptosis-mediated neurotoxicity induced by beta-amyloid and PrP fragments. Mol Chem Neuropathol
20 *28*, 163-71.

Garcia-Calvo, M., Peterson, E. P., Leiting, B., Ruel, R., Nicholson, D. W., and Thornberry, N. A. (1998). Inhibition of human caspases by peptide-based and macromolecular inhibitors. J Biol Chem *273*, 32608-13.
25
Haass, C., Lemere, C. A., Capell, A., Citron, M., Seubert, P., Schenk, D., Lannfelt, L., and Selkoe, D. J. (1995). The Swedish mutation causes early-onset Alzheimer's disease by beta- secretase cleavage within the secretory pathway. Nat Med *1*, 1291-6.

30 Haass, C., and Selkoe, D. J. (1993). Cellular processing of beta-amyloid precursor protein and the genesis of amyloid beta-peptide. Cell *75*, 1039-42.

Holcomb, L., Gordon, M. N., McGowan, E., Yu, X., Benkovic, S., Jantzen, P., Wright, K., Saad, I., Mueller, R., Morgan, D., Sanders, S., Zehr, C., O'Campo, K.,
35 Hardy, J., Prada, C. M., Eckman, C., Younkin, S., Hsiao, K., and Duff, K. (1998).

Accelerated Alzheimer-type phenotype in transgenic mice carrying both mutant amyloid precursor protein and presenilin 1 transgenes. Nat Med *4*, 97-100.

Kim, T. W., Pettingell, W. H., Jung, Y. K., Kovacs, D. M., and Tanzi, R. E. (1997). Alternative Cleavage of Alzheimer-Associated Presenilins During Apoptosis by a Caspase-3 Family Protease. Science *277*, 373-6.

Kuida, K., Zheng, T. S., Na, S., Kuan, C., Yang, D., Karasuyama, H., Rakic, P., and Flavell, R. A. (1996). Decreased apoptosis in the brain and premature lethality in CPP32- deficient mice. Nature *384*, 368-72.

LaFerla, F. M., Tinkle, B. T., Bieberich, C. J., Haudenschild, C. C., and Jay, G. (1995). The Alzheimer's A beta peptide induces neurodegeneration and apoptotic cell death in transgenic mice. Nat Genet *9*, 21-30.

LeBlanc, A. (1995). Increased production of 4 kDa amyloid beta peptide in serum deprived human primary neuron cultures: possible involvement of apoptosis. J Neurosci *15*, 7837-46.

LeBlanc, A. C., Xue, R., and Gambetti, P. (1996). Amyloid precursor protein metabolism in primary cell cultures of neurons, astrocytes, and microglia. J Neurochem *66*, 2300-10.

Li, W. P., Chan, W. Y., Lai, H. W., and Yew, D. T. (1997). Terminal dUTP nick end labeling (TUNEL) positive cells in the different regions of the brain in normal aging and Alzheimer patients. J Mol Neurosci *8*, 75-82.

Loo, D. T., Copani, A., Pike, C. J., Whittemore, E. R., Walencewicz, A. J., and Cotman, C. W. (1993). Apoptosis is induced by beta-amyloid in cultured central nervous system neurons. Proc Natl Acad Sci U S A *90*, 7951-5.

Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998). An induced proximity model for caspase-8 activation. J Biol Chem *273*, 2926-30.

Nicholson, D. W., Ali, A., Thornberry, N. A., Vaillancourt, J. P., Ding, C. K., Gallant, M., Gareau, Y., Griffin, P. R., Labelle, M., Lazebnik, Y. A., and et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature *376*, 37-43.

Nicholson, D. W., and Thornberry, N. A. (1997). Caspases: killer proteases. Trends Biochem Sci *22*, 299-306.

Pulsinelli, W. A., Brierley, J. B., and Plum, F. (1982). Temporal profile of neuronal damage in a model of transient forebrain ischemia. Ann Neurol *11*, 491-8.

Pulsinelli, W. A., and Buchan, A. M. (1988). The four-vessel occlusion rat model: method for complete occlusion of vertebral arteries and control of collateral circulation. Stroke *19*, 913-4.

Rano, T. A., Timkey, T., Peterson, E. P., Rotonda, J., Nicholson, D. W., Becker, J. W., Chapman, K. T., and Thornberry, N. A. (1997). A combinatorial approach for determining protease specificities: application to interleukin-1beta converting enzyme (ICE). Chem Biol *4*, 149-55.

Rotonda, J., Nicholson, D. W., Fazil, K. M., Gallant, M., Gareau, Y., Labelle, M., Peterson, E. P., Rasper, D. M., Ruel, R., Vaillancourt, J. P., Thornberry, N. A., and Becker, J. W. (1996). The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat Struct Biol *3*, 619-25.

Russo, T., Faraonio, R., Minopoli, G., De Candia, P., De Renzis, S., and Zambrano, N. (1998). Fe65 and the protein network centered around the cytosolic domain of the Alzheimer's beta-amyloid precursor protein. FEBS Lett *434*, 1-7.

Selkoe, D. J., Yamazaki, T., Citron, M., Podlisny, M. B., Koo, E. H., Teplow, D. B., and Haass, C. (1996). The role of APP processing and trafficking pathways in the formation of amyloid beta-protein. Ann N Y Acad Sci *777*, 57-64.

Sisodia, S. S., and Price, D. L. (1995). Role of the beta-amyloid protein in Alzheimer's disease. Faseb J *9*, 366-70.

Smale, G., Nichols, N. R., Brady, D. R., Finch, C. E., and Horton, W. E., Jr. (1995). Evidence for apoptotic cell death in Alzheimer's disease. Exp Neurol *133*, 225-30.

5   Srinivasula, S. M., Ahmad, M., Fernandes-Alnemri, T., and Alnemri, E. S. (1998). Autoactivation of procaspase-9 by Apaf-1-mediated oligomerization. Mol Cell *1*, 949-57.

Su, J. H., Deng, G., and Cotman, C. W. (1997). Bax protein expression is increased in
10   Alzheimer's brain: correlations with DNA damage, Bcl-2 expression, and brain pathology. J Neuropathol Exp Neurol *56*, 86-93.

Tewari, M., Quan, L. T., O'Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995). Yama/CPP32 beta, a
15   mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase. Cell *81*, 801-9.

Thornberry, N. A., Rano, T. A., Peterson, E. P., Rasper, D. M., Timkey, T., Garcia-Calvo, M., Houtzager, V. M., Nordstrom, P. A., Roy, S., Vaillancourt, J. P.,
20   Chapman, K. T., and Nicholson, D. W. (1997). A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem *272*, 17907-11.

Vito, P., Ghayur, T., and D'Adamio, L. (1997). Generation of anti-apoptotic
25   presenilin-2 polypeptides by alternative transcription, proteolysis, and caspase-3 cleavage. J Biol Chem *272*, 28315-20.

Wang, R., Sweeney, D., Gandy, S. E., and Sisodia, S. S. (1996). The profile of soluble amyloid beta protein in cultured cell media. Detection and quantification of amyloid
30   beta protein and variants by immunoprecipitation-mass spectrometry. J Biol Chem *271*, 31894-902.

Xu, D. G., Crocker, S. J., Doucet, J. P., St-Jean, M., Tamai, K., Hakim, A. M., Ikeda, J. E., Liston, P., Thompson, C. S., Korneluk, R. G., MacKenzie, A., and Robertson,

G. S. (1997). Elevation of neuronal expression of NAIP reduces ischemic damage in the rat hippocampus. Nat Med *3*, 997-1004.

Yang, X., Chang, H. Y., and Baltimore, D. (1998). Autoproteolytic activation of pro-
5   caspases by oligomerization. Mol Cell *1*, 319-25.

Zhang, L., Zhao, B., Yew, D. T., Kusiak, J. W., and Roth, G. S. (1997). Processing of Alzheimer's amyloid precursor protein during H_2O_2-induced apoptosis in human neuronal cells. Biochem Biophys Res Commun *235*, 845-8.

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All references cited herein are hereby incorporated by reference in their entirety.

WHAT IS CLAIMED IS:

1.   An antibody that recognizes a neo-epitope created or exposed following a caspase mediated cleavage of APP and APLP.

5

2.   The antibody of claim 1, wherein said antibody is raised with an antigenic determinant having a consensus sequence of a caspase cleavage site of at least four amino acids, said consensus sequence comprising V/DXXD.

10   3.   An antibody in accordance with claim 1, wherein the APP is cleaved by a caspase selected from caspase 3, caspase 6 and caspase 8.

4.   An antibody in accordance with claim 3, wherein the APP and APLP is cleaved by caspase 3.

15

5.   An antibody in accordance with claim 4, wherein the neo-epitope created or exposed following a caspase mediated cleavage of APP is cleaved by caspase 3.

20   6.   An antibody in accordance with claim 5, wherein the APP comprises three caspase 3 cleavage sites designated 197, 219 and 720 based upon the amino acid numbering of APP^{751}.

7.   An antibody in accordance with claim 6 capable of binding to
25 the neo-epitope created or exposed following the caspase 3 cleavage at the designated cleavage site, 720.

8.   An antibody raised with an antigenic determinant comprising a peptide of at least four amino acid residues and containing the caspase consensus
30 sequence V/DXXD.

9.   An antibody in accordance with claim 8 raised with the antigenic determinant comprising a peptide having the amino acid sequence APP^{714} to APP^{720}.

10.   An antibody in accordance with claim 9, wherein the antigenic determinant is coupled to an antigenicity enhancer.

5   11.   An antibody in accordance with claim 10, wherein the coupled antigenic determinant comprises KLH-cysteine- APP^{714} to APP^{720}.

12.   An antibody in accordance with claim 1, wherein said antibody is a polyclonal.

10   13.   An antibody in accordance with claim 1, wherein said antibody is a monoclonal.

14.   An anti-serum comprised of an antibody that is capable of binding to a neo-epitope, exposed following a caspase mediated cleavage of APP and 15   APLP.

15.   A method for generating an antibody capable of binding to a neo-epitope, created or exposed following a caspase mediated cleavage of APP and APLP comprising:

20   immunizing a mammal with a peptide having at least four amino acid residues and containing the caspase consensus sequence V/DXXD to provide antisera containing antibodies against said peptide;

collecting said antisera; and

selecting for an antibody specific to a caspase generated neo-epitope.

25

16.   A method for detecting or characterizing the level of caspase mediated apoptosis in a mammalian patient, comprising:

obtaining a tissue sample from a patient suspected of having neuronal apoptosis;

30   contacting the sample with an antibody as described in claim 1 to detect the presence or amount of a caspase-cleaved APP and APLP;

and comparing the amount of caspase-cleaved APP and APLP to a standard.

17.   A method in accordance with claim 16, wherein the tissue sample is selected from blood, plasma and cerebrospinal fluid.

18.   A method in accordance with claim 17, wherein the level of caspase mediated cleavage is an indication of neuronal apoptosis due to conditions comprising, a brain or spinal cord injury, and in neurodegenerative disease selected from the group consisting of: Alzheimer's disease, Huntington's disease, Parkinson's disease, amyotrophic lateral sclerosis, progressive multiple sclerosis, head trauma, prion related conditions, Creutzfeldt-Jacob disease, spongiform encephalopathy, Friedreich's ataxia, fatal familial insomnia, Pelizaeus-Merzbacher disease, schizophrenia, dentatorubropallidoluysian atrophy, spinocerebellar atrophy type 3, spinal bulbar muscular atrophy, and stroke.

19.   A method for detecting or characterizing the level of caspase mediated apoptosis in a tissue sample, comprising:

obtaining a tissue sample;

contacting the sample with an antibody as described in claim 1 to detect the presence or amount of caspase-cleaved APP;

and comparing the amount of caspase-cleaved APP to a standard.

20.   A method in accordance with claim 19, further comprising contacting the tissue sample with a compound that is suspected of modulating the level of neuronal apoptosis.

21.   A method in accordance with claim 20, wherein the tissue is neuronal tissue.

22.   A method in accordance with claim 20, wherein the tissue is not neuronal tissue and can be selected from blood, plasma and cerebrospinal fluid.

23.   A detection kit designed to detect caspase mediated APP and APLP cleavage products comprising at least one antibody as defined in claim 1.

24.   An antigenic determinant comprising the amino acid residues APP^{714} to APP ^{720} based on the numbering of APP751 .

- 41 -

25. An antigenic determinant in accordance with claim 24 further coupled to an antigenicity enhancer, comprising KLH-cysteine- APP^{714} to APP^{720}.
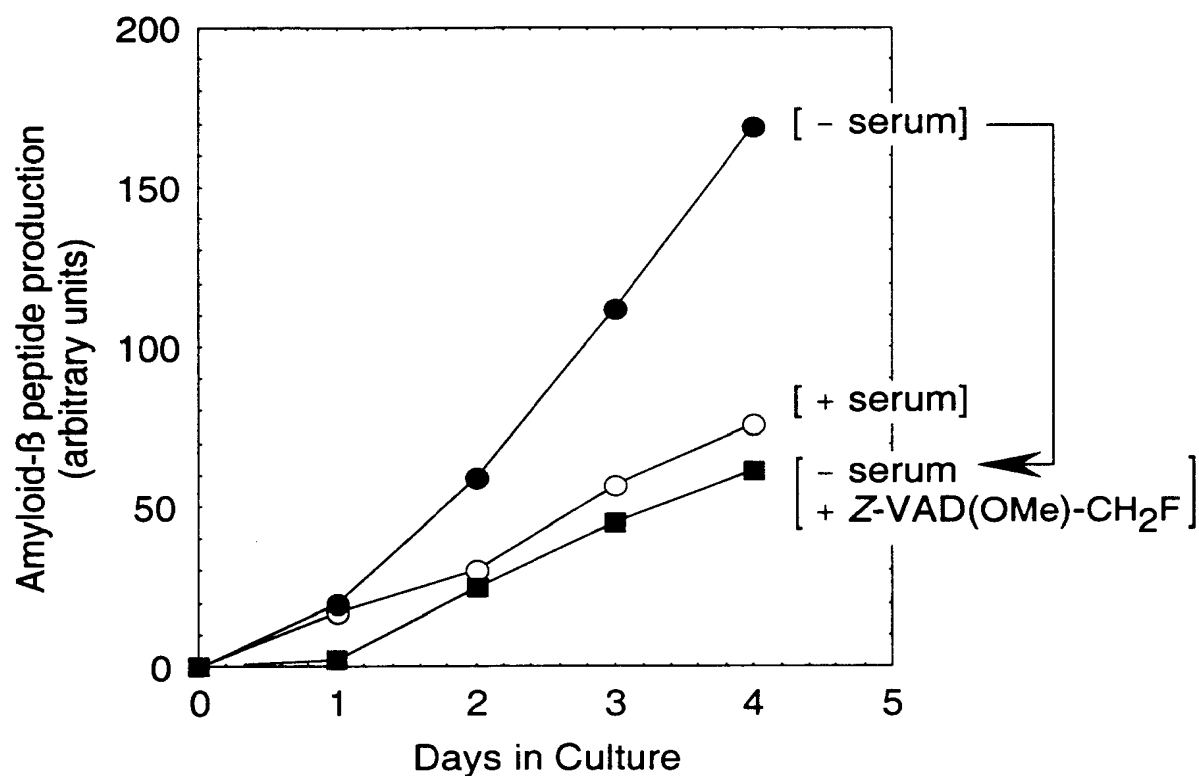
FIG.1A
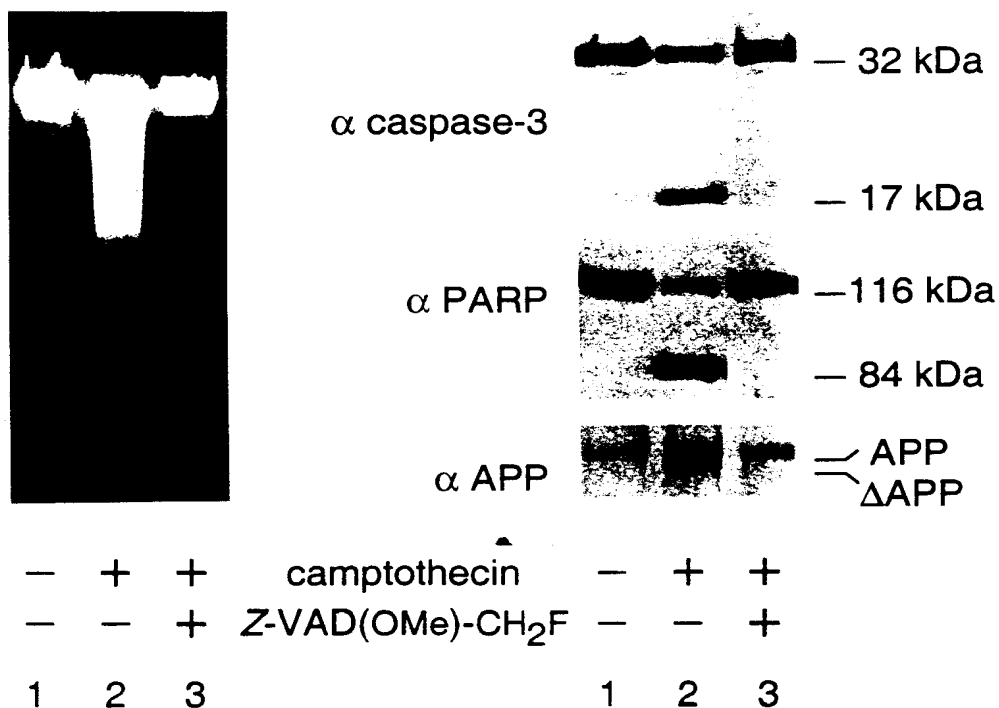
FIG.1B
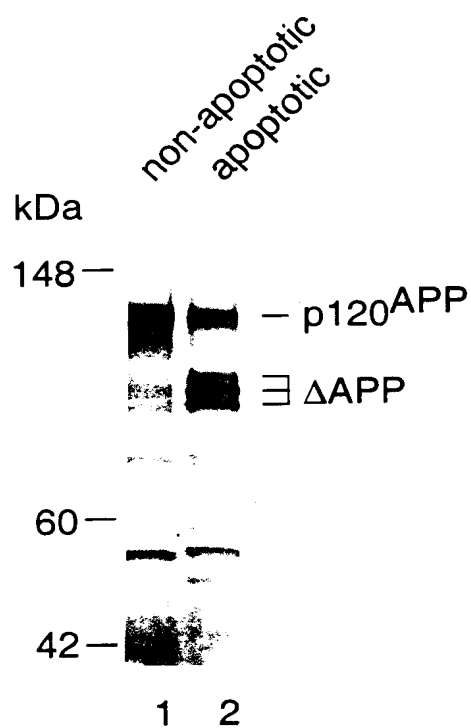
FIG.1C
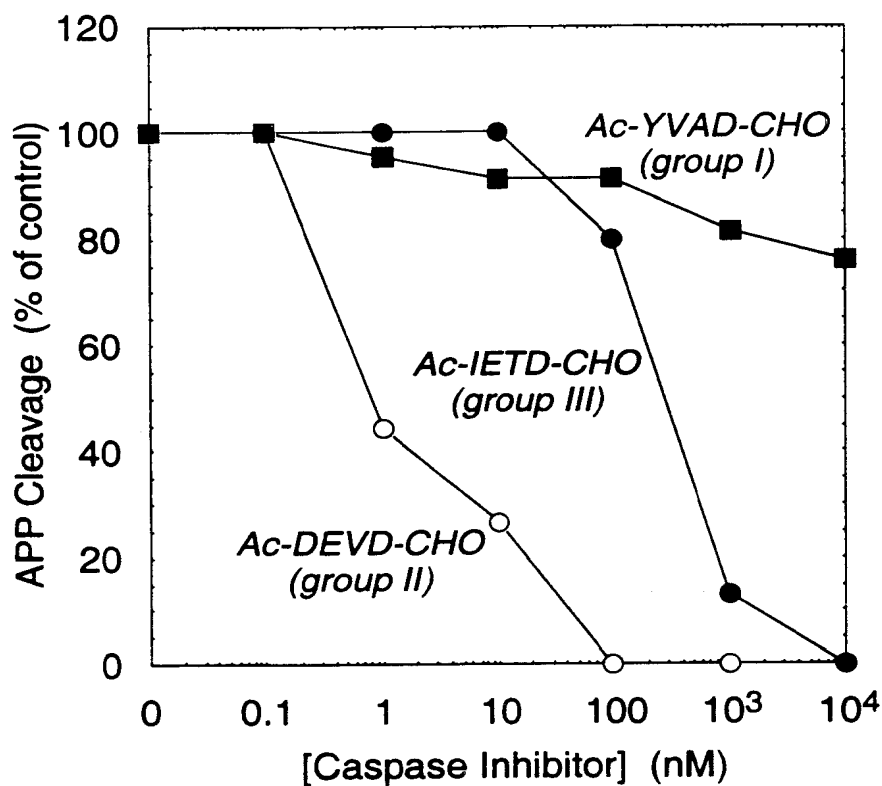
FIG.1D
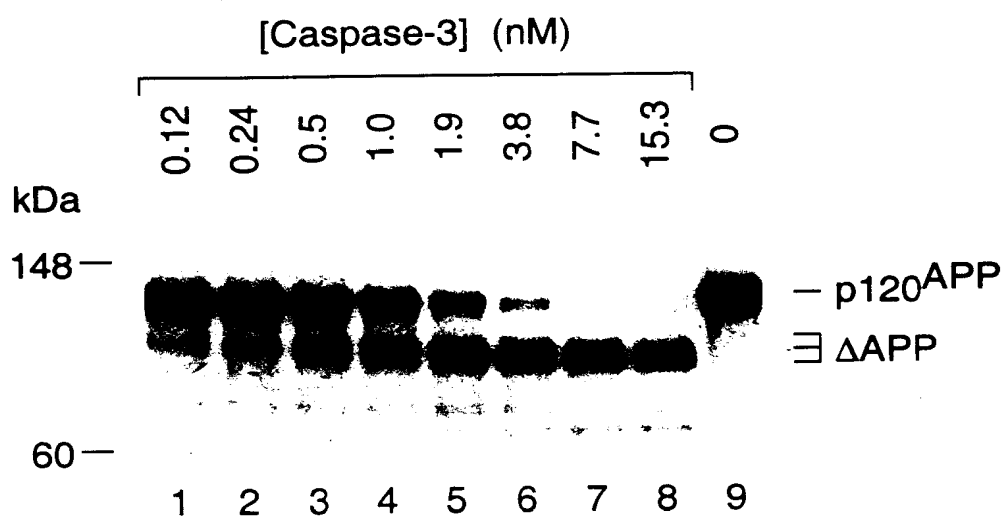
FIG.1E

$$k_{cat}/K_m = 5.14 \times 10^5 \ M^{-1}s^{-1}$$

FIG.2A

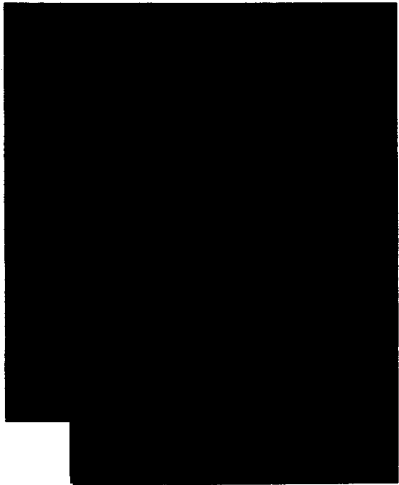FIG.2B

FIG.3A

FIG. 3B

FIG.4C

FIG.4B
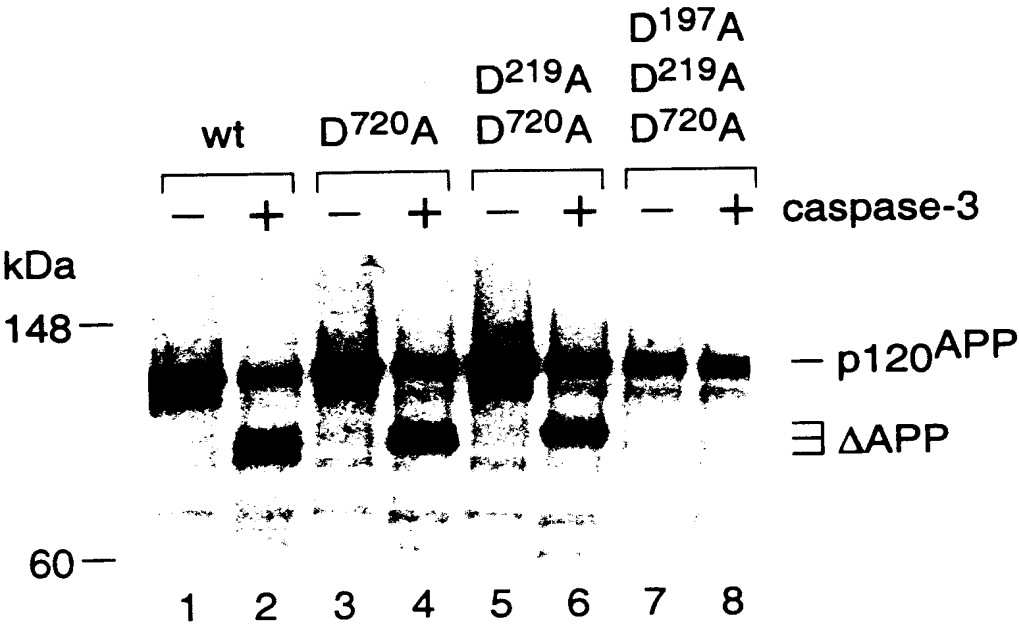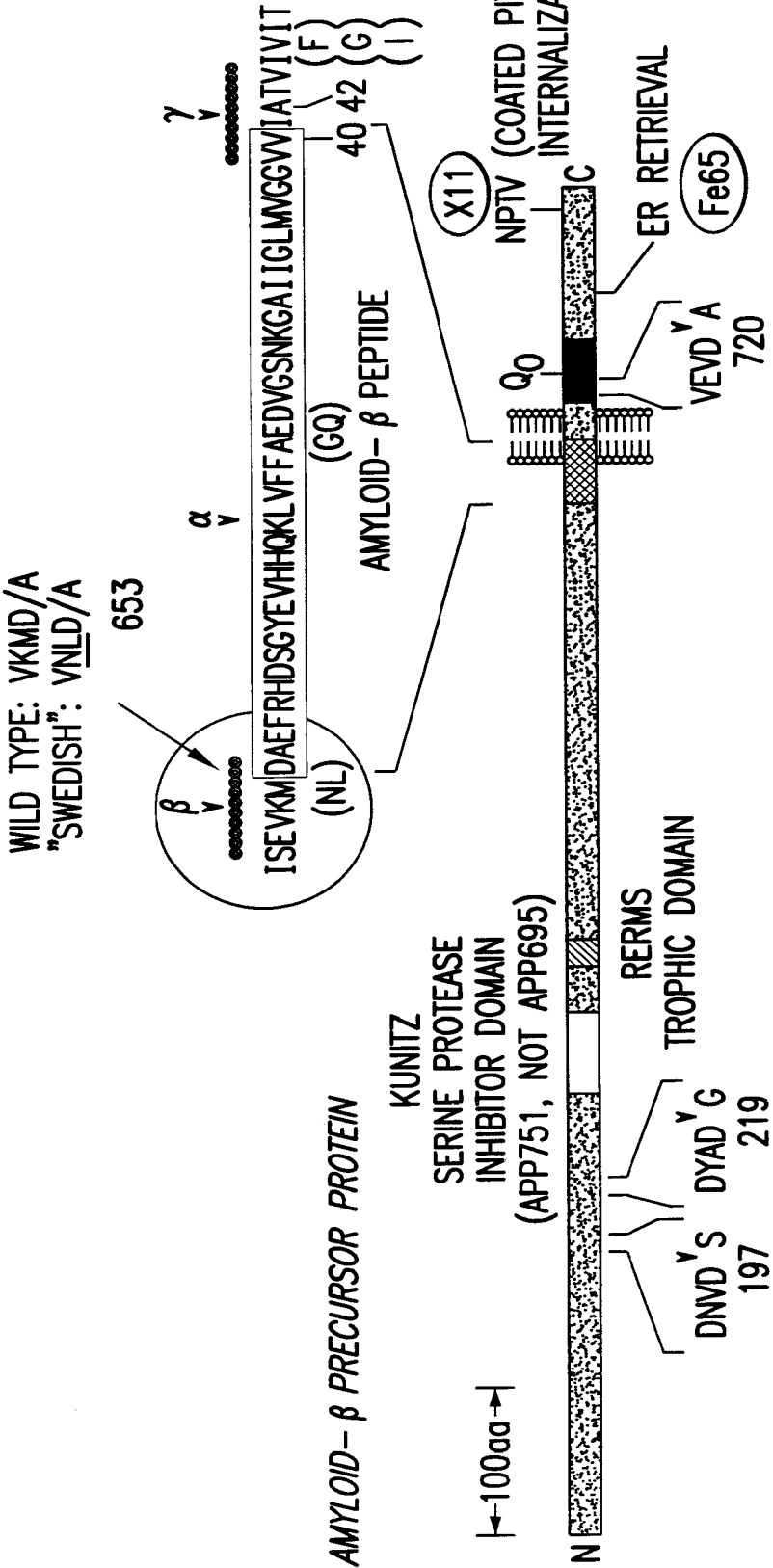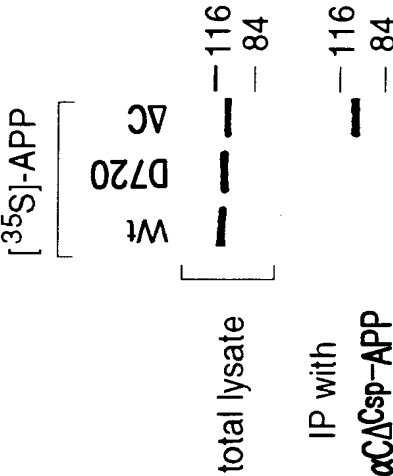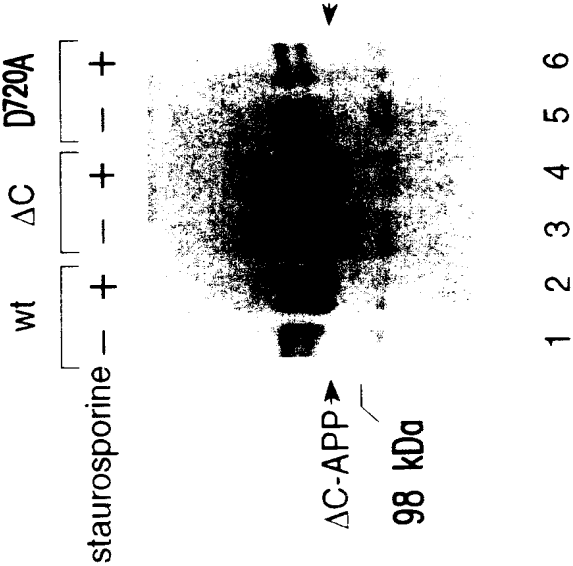
FIG.4A
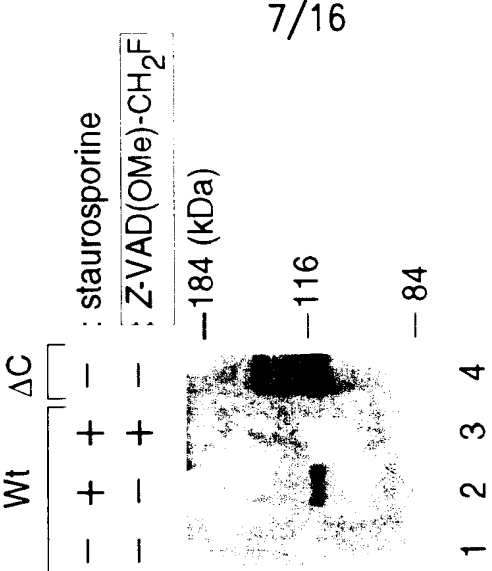
TUNEL,
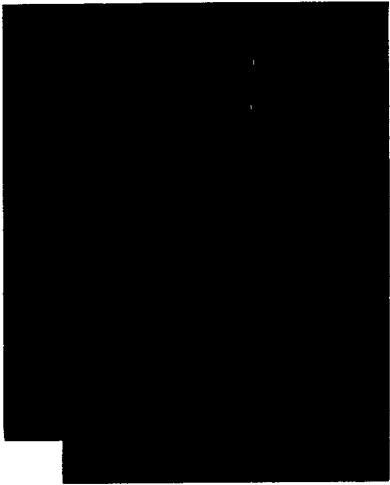αCsp-3(MF397)

camptothecin

FIG.4E
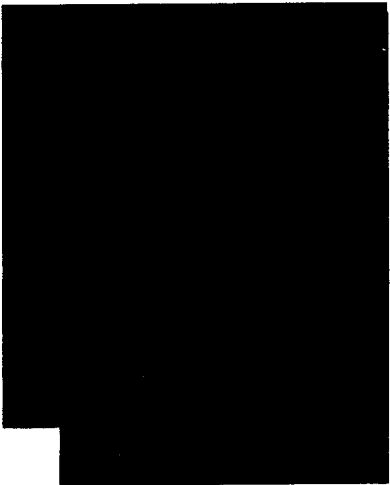
TUNEL,
αC Csp-APP
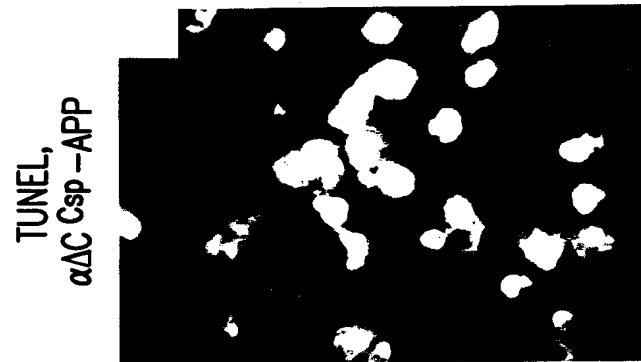
control

FIG.4G

TUNEL,
αCsp-3(MF397)

control

FIG.4D

TUNEL,
αCsp-3(MF397)

camptothecin,
Z-VAD(OMe)-CH2F

FIG.4F

camptothecin

FIG.4H

camptothecin,
Z-VAD(OMe)-CH$_2$F

FIG.4I

FIG.5A
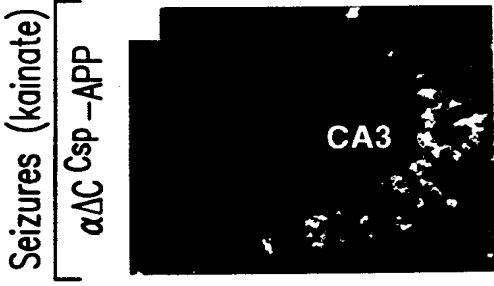
FIG.5B

FIG.5C

FIG.5D

FIG.5E
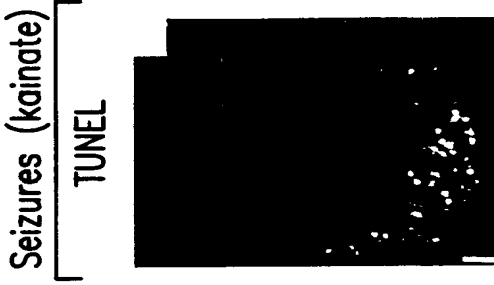
FIG.5F
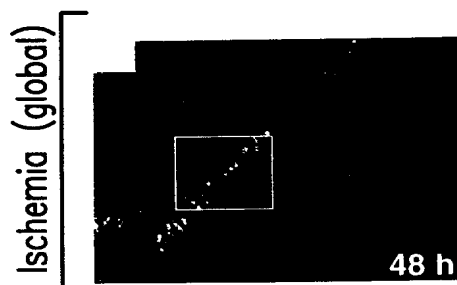
FIG.5G

FIG.5H

FIG.5I

FIG.5J
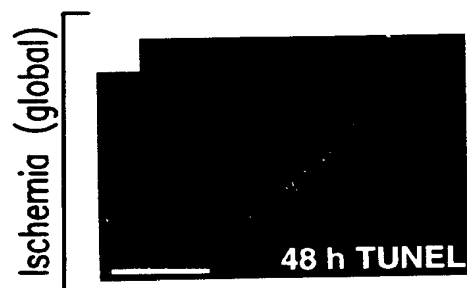
FIG.5K
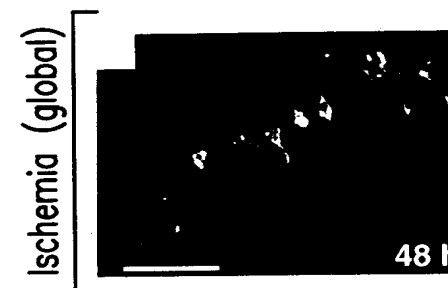
FIG.5L
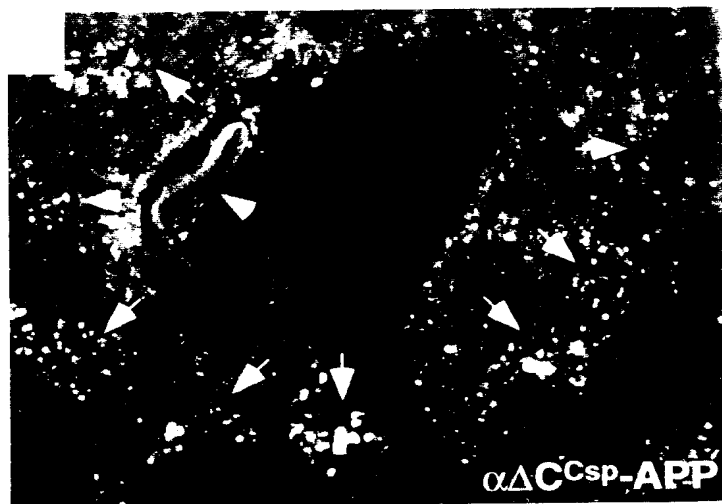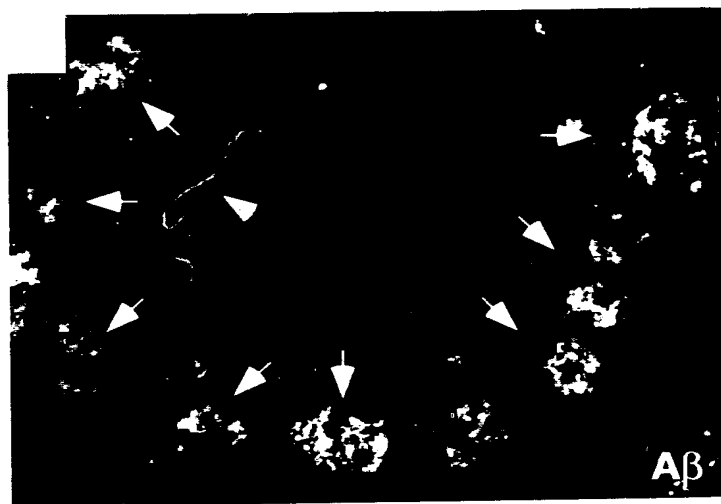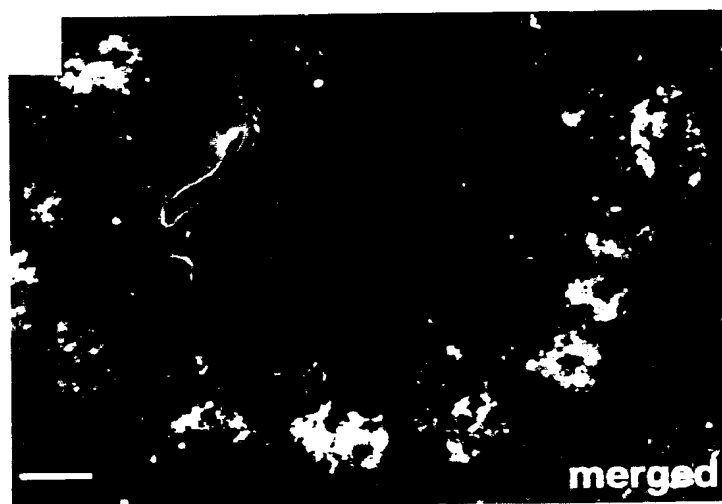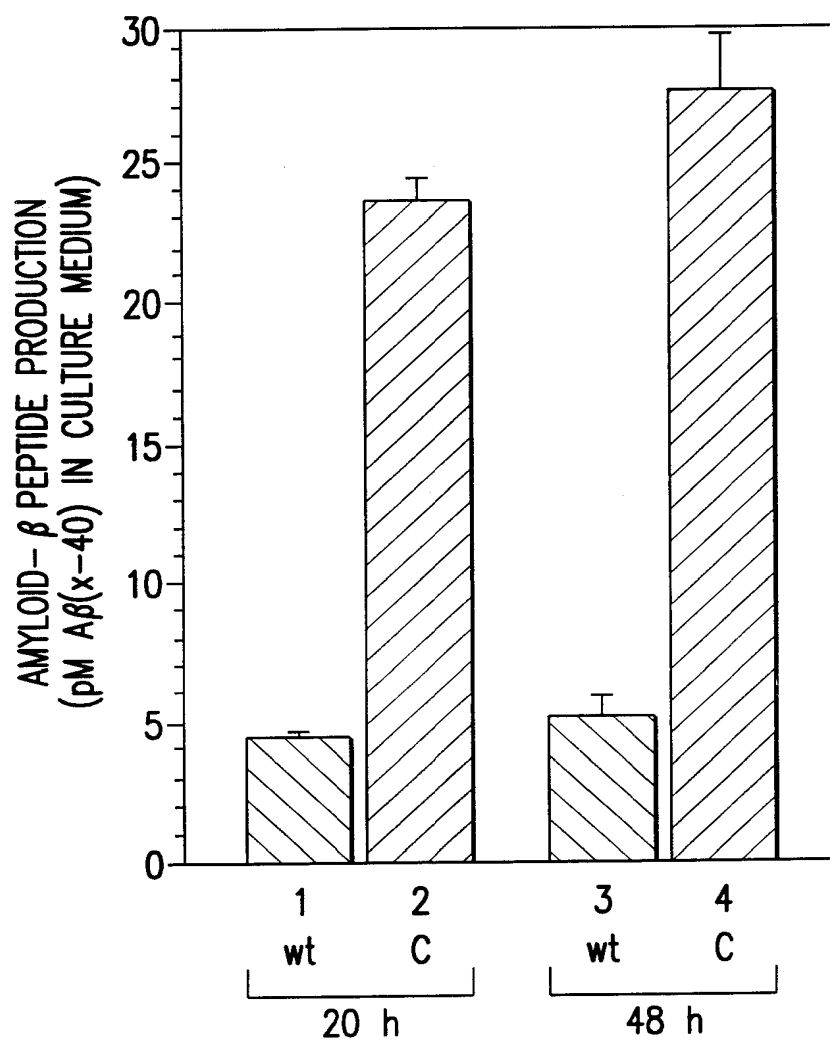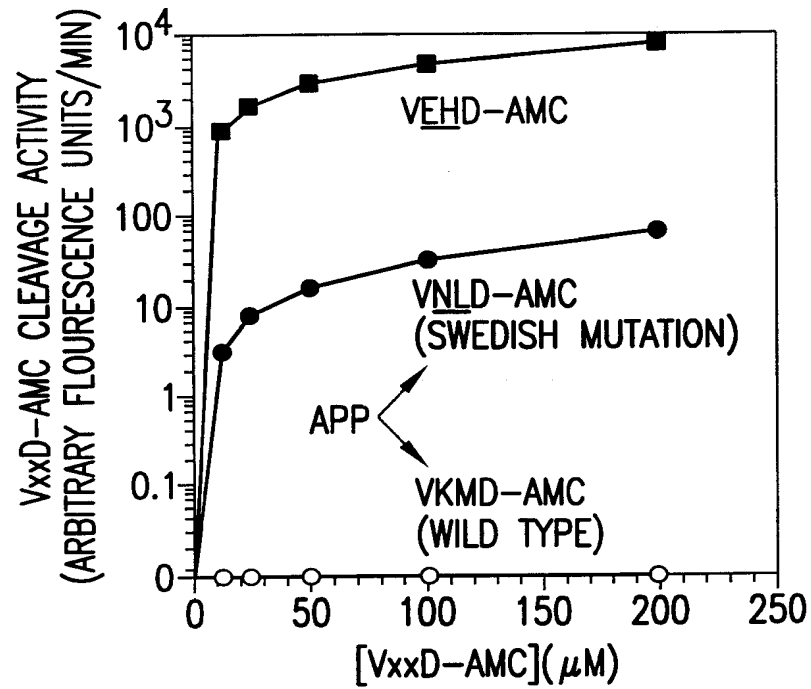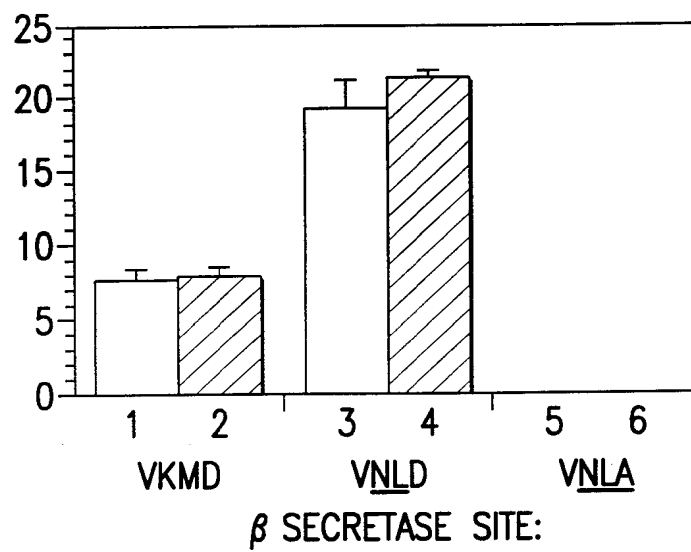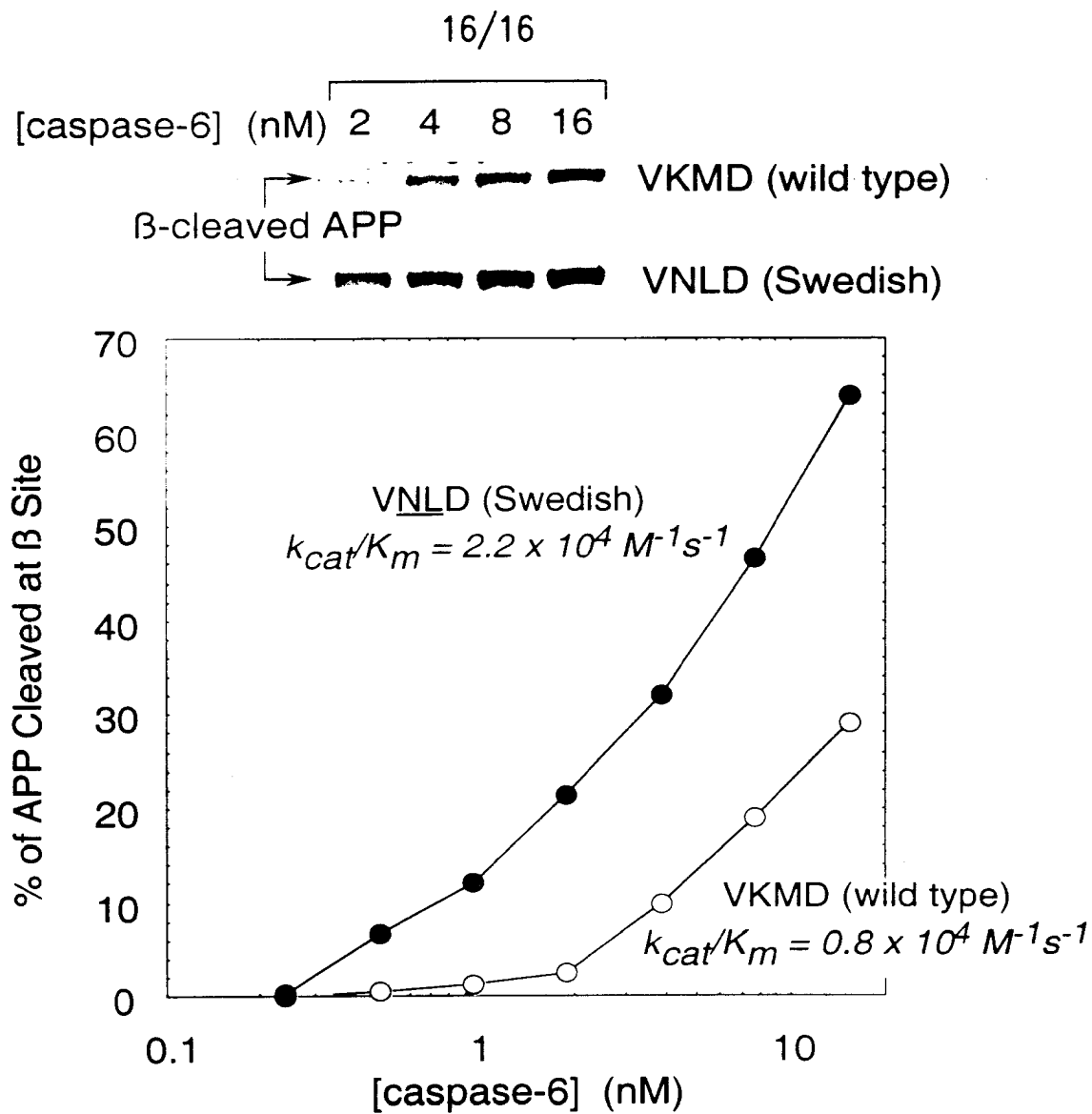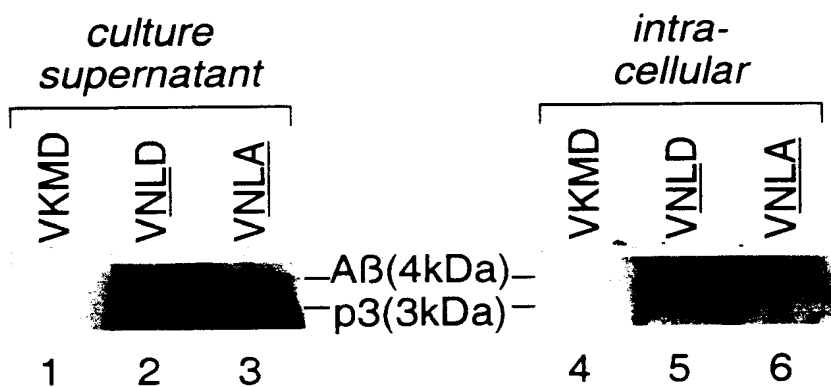
FIG.6A

FIG.6B

FIG.6C

FIG.7

FIG.8A

FIG.8B

16/16

[caspase-6] (nM) 2   4   8   16

→ ········ ▬▬ ▬▬ ▬▬   VKMD (wild type)

β-cleaved APP

↳ ▬▬ ▬▬ ▬▬ ▬▬   VNLD (Swedish)

VNLD (Swedish)
$k_{cat}/K_m = 2.2 \times 10^4\ M^{-1}s^{-1}$

VKMD (wild type)
$k_{cat}/K_m = 0.8 \times 10^4\ M^{-1}s^{-1}$

% of APP Cleaved at β Site

[caspase-6] (nM)

FIG.8C

culture
supernatant

intra-
cellular

VKMD VNLD VNLA   VKMD VNLD VNLA

—Aβ(4kDa)—
—p3(3kDa)—

1   2   3   4   5   6

FIG.8D

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7   C07K16/18   G01N33/68   A61K39/00   C07K14/47

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7   C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 21590 A (CEPHALON INC) 22 May 1998 (1998-05-22)	8
Y	abstract page 3, line 7 - line 16 page 4, line 15 - line 29 page 10, line 12 - line 27 page 14; example 1	1-7,9-25
X	WO 98 52597 A (CHIRON CORP ;KOTHS KIRSTON (US); KOTHAKOTA SRINIVAS (US); REINHART) 26 November 1998 (1998-11-26)	8
Y	abstract page 3, paragraph 2 - paragraph 3 page 20, paragraph 2 -page 21, paragraph 1	1-7,9-25
	-/--	

[X] Further documents are listed in the continuation of box C.   [X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
16 August 2000	15. 09. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Montrone, M

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
Y	GERVAIS F ET AL: "Involvement of caspases in proteolytic cleavage of Alzheimer's beta amyloid precursor protein and amyloidogenic beta-peptide formation." SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 24, no. 1-2, 1998, page 206 XP000917593 28th Annual Meeting of the Society for Neuroscience, Part 1;Los Angeles, California, USA; November 7-12, 1998 ISSN: 0190-5295 abstract	1-7,9-25
Y	WEIDEMANN ANDREAS ET AL: "Proteolytic processing of the Alzheimer's disease amyloid precursor protein within its cytoplasmic domain by caspase-like proteases." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 274, no. 9, 26 February 1999 (1999-02-26), pages 5823-5829, XP002145058 ISSN: 0021-9258 abstract figure 1 page 5825, column 1, paragraph 2 -page 5826, column 2, paragraph 2 page 5827, column 2, paragraph 3 -page 5828, column 1, paragraph 1	1-7,9-25
Y	BARNES N Y ET AL: "Caspase 3 cleaves amyloid precursor protein in dying motoneurons." SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 24, no. 1-2, 1998, page 1786 XP000917592 28th Annual Meeting of the Society for Neuroscience, Part 2;Los Angeles, California, USA; November 7-12, 1998 ISSN: 0190-5295 abstract	1-7,9-25
Y	BARNES NATALIE Y ET AL: "Increased production of amyloid precursor protein provides a substrate for caspase-3 in dying motoneurons." JOURNAL OF NEUROSCIENCE, vol. 18, no. 15, 1 August 1998 (1998-08-01), pages 5869-5880, XP000917584 ISSN: 0270-6474 abstract page 5874, column 2, paragraph 2 -page 5875, column 1, paragraph 1 figure 7	1-7,9-25

-/--

1

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	THORNBERRY NANCY A ET AL: "A combinatorial approach defines specificities of members of the caspase family and granzyme B: Functional relationships established for key mediators of apoptosis." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 272, no. 29, 1997, pages 17907-17911, XP002145059 ISSN: 0021-9258 abstract figures 1-3	1-14
P,X	GERVAIS FRANCOIS G ET AL: "Involvement of caspases in proteolytic cleavage of Alzheimer's amyloid-beta precursor protein and amyloidogenic Abeta peptide formation." CELL, vol. 97, no. 3, 30 April 1999 (1999-04-30), pages 395-406, XP000919376 ISSN: 0092-8674 abstract page 398, column 1, paragraph 2 -page 401, column 1, paragraph 1 figure 4 page 401, column 2, paragraph 4 -page 403, column 1, paragraph 1 figure 8 page 404, column 1, paragraph 2	1-25

1

INTERNATIONAL SEARCH REPORT

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 15 to 22 (partially) are directed to a method of treatment or surgery of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
   because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest
[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9821590	A	22-05-1998	US	6048703 A	11-04-2000
			AU	5169998 A	03-06-1998
			EP	0975971 A	02-02-2000
WO 9852597	A	26-11-1998	AU	7578498 A	11-12-1998
			EP	1003543 A	31-05-2000